View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Technische Universität Dresden: Qucosa

Production of prostaglandin E_2 and thromboxane A_2 by rat liver macrophages and involvement of nitric oxide and cytokines in mediator pathways under inflammatory conditions

D I S S E R T A T I O N

zur Erlangung des akademischen Grades

Doctor rerum naturalium
(Dr. rer. nat.)

vorgelegt
der Fakultät Mathematik und Naturwissenschaften
der Technischen Universität Dresden

von
Yevgeniya Bezugla

geboren am 05.05.1976 in Pawlograd

Gutachter:

1. Prof. Dr. rer. nat. habil. Karl-Heinz van Pée
2. Prof. Dr. rer. nat. Peter Dieter
3. Prof. Dr. rer. nat. Gerhard Paul Püschel

Eingereicht am: 17.07.2007

Tag der Verteidigung: 08.01.2008

List of publications

Bezugla, Y., Kolada, A., Kamionka, S., Bernard, B., Scheibe, R., Dieter, P. COX-1 and COX-2 contribute differentially to the LPS-induced release of PGE_2 and TxA_2 in liver macrophages. Prostaglandins Other Lipid Mediat. 2006 Mar;79(1-2):93-100.

Dieter P., Scheibe R., Bezugla Y., Matthe E., Schuch S., Treffkorn L., Bernard B., Kamionka S., Kolada A. The regulatory role of prostaglandin E_2 in liver (patho) physiology is controlled at its site of synthesis and its action on the receptors. Comp Hepatol. 2004 Jan 14;3 Suppl 1:S35.

Schade S, Bezugla Y, Kolada A, Kamionka S, Scheibe R, Dieter P. Diverse functional coupling of cyclooxygenase 1 and 2 with final prostanoid synthases in liver macrophages. Biochem Pharmacol. 2002 Oct 15;64(8):1227-32.

Conference contributions:

Bezugla Y., Kolada A., Kamionka S., Bernard B., Scheibe R., Dieter P. Autocrine effect of cytokines on the release of inflammatory mediators in rat Kupffer cells. "21. Jahrestagung der Deutschen Arbeitsgemeinschaft zum Studium der Leber", Januar 2005.

Bezugla Y., Kolada A., Kamionka S., Bernard B., Scheibe R., Dieter P. Interaction of nitric oxide and prostanoids in Kupffer cells during inflammation. "20. Jahrestagung der Deutschen Arbeitsgemeinschaft zum Studium der Leber", Januar 2004.

Bezugla Y., Scheibe R., Kolada A, Kamionka S., Dieter P. Autocrine effect of cytokines on prostanoid synthesis and nitric oxide release in rat Kupffer cells. in "ELSO – 2003 meeting", September 2003.

Bezugla Y., Scheibe R., Kolada A, Kamionka S., Bernard B., Dieter P. Effect of Cytokines (IL-1ß, TNF-α, IL-10) on the Release of Prostanoids and Nitric Oxide in Kupffer Cells. "8th International Conference Eicosanoids & Other Bioactive Lipids in Cancer, Inflammation & Related Diseases", September 2003.

Bezugla Y., Scheibe R., Dieter P. Dexamethasone influences on the prostanoid synthesis pathway in LPS-activated liver macrophages, "19. Jahrestagung der Deutschen Arbeitsgemeinschaft zum Studium der Leber", Januar 2003.

Abbreviations

AA	Arachidonic acid
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
Aca/Bis	Acrylamid/Bisacrylamid
AMP	Adenosine monophosphate
BCA	Bichinonic acid
BSA	Bovine serum albumin
cDNA	copy DNA
COX	Cyclooxygenase
cPGES	Cytosolic prostaglandin E_2 synthase
cPLA$_2$	Cytoplasmic phospholipase A_2
DAB	3,3-diaminobenzidine
DAG	Diacylglycerol
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	Diethylpyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EC	Endothelial cell
E. coli	Escherichia coli
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EC number	Enzyme Commission number
EDCI	N-ethyl, N'-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA	Ehylenediaminetetraacetic acid
EGTA	Bis(2-aminoethyl)ethyleneglycoltetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPR	Eelectron paramagnetic resonance spectroscopy
FCS	Fetal calf serum
GSH	Glutathione
GST	Glutathione S-transferase
h	Hour
HBSS	Hanks Balanced Salt Solution
HSC	Hepatic Stellate cells
HEK	Human embryonic kidney
HETE	Hydroxyeicosatrienoic acid
HHT	hydiroxy-5,8,10-heptadecatrienoic acid
HGF	Hepatocyte Growth Factor
HRP	Horseradish Peroxidase
IFN	Interferon

IL	Interleukin
IP	Inositolphosphate
kb	kilobases
KC	Kupffer cell
KC/GRO	Keratinocyte/melanoma growth stimulating factor
kDa	Kilo Dalton
K_M	Michaelis constant
L-NMMA	N^G-Monomethyl-L-Arginin
L-NIL	L-N^6-(1-Iminoethyl)lysine, HCl
LPS	Lipopolysaccaride
LTA	Leukotriene A_4
MAPEG	Membrane-associated proteins in eicosanoid and gluthatione metabolism
MAPK	Mitogen-activated protein kinase
MIP	Macrophage inflammatory protein
MC	Monocyte
MCD	Magnetic circular dichroism spectroscopy
MCP	Monocyte chemoattractant protein
MDA	Malondialdehyde
MP	Macrophage
min	Minute
MOPS	3-(N-Morpholino)-propanesulfonic acid
mPGES	Microsomal prostaglandin E_2 synthase
mRNA	Messenger RNA
MW	Molecular weight
NCS	Newborn calf serum
NO	Nitric oxide
NOS	Nitric oxide synthase
NPC	Nonparenhymal cells
ON	Overnight
PAF	Platelet-activating factor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered Saline
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PGES	Prostaglandin E_2 synthase
PKC	Protein kinase C
PL	Phospholipase
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethyl sulfonylfluoride
RES	Reticuloendothelial system
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotation per minute

RPMI medium	Roswell Park Memorial Institute medium
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
Sulfo-NHS	N-Hydroxysulfosuccinimide sodium salt
SNAP	S-Nitroso-N-acetylpenicillamine
sPLA$_2$	Secretory phospholipase A_2
TBE	Tris-borat-EDTA buffer
TBS	Tris buffered saline
TBS-T	Tris Buffered Saline with Tween 20
TCA	Trichloroacetic acid
TEMED	N, N, N', N' - tetramethylethylendiamine
TGF-β	Transforming growth factor -β
TNFα	Tumor necrosis factor α
Tx	Thromboxane
TxAS	Thromboxane A_2 synthase
U	Unit
UV-VIS	Ultraviolet-visible spectroscopy
v/v	Volume percentage (volume per volume)
w/v	Weight-volume percentage (weight per volume)

CONTENTS

1. INTRODUCTION

The process of wound healing in the liver, in contrast to other organs, leads often to a marked deterioration associated with portal hypertension and hepatocyte dysfunction. The development of hepatic fibrosis begins with infiltration of the space of Disse with increased amounts of collagens, glycoproteins and progresses up to nodule formation and cirrhosis (Davis *et al.*, 1996). The liver damage is provoked by various chemical and biological agents, for instance by ethanol, oxidative stress, viruses and bacterial infections (Hoek *et al.*, 2002). Liver transplantation, hyvopolemic shock or trauma also often entails the inflammation (Farzaneh-Far *et al.*, 2001).

The development of inflammation in the liver involves the close cooperation between parenchymal cells - hepatocytes and non-parenchymal (sinusoidal) cells (NPC).

1.1. Rat liver macrophages and their role in liver (patho)physiology

1.1.1. Non-parenchymal cells

Non-parenchymal liver cells (sinusoidal) cells represent a functional division between hepatocytes and the blood (Fig.1.1.).

They include several types of cells (Table 1.1.): sinusoidal endothelial cells (EC) which compose the liver capillary wall; Kupffer cells (KC) which are the resident liver macrophages; hepatic Stellate (fat-storing, Ito) cells (HSC) which are located in the perisinusoidal space of Disse and are the main hepatic source of extracellular matrix components; Pit cells which are the kind of granular lymphocytes (Bouwens *et al.*, 1992; Arias *et al.*, 1982; Decker, 1987).

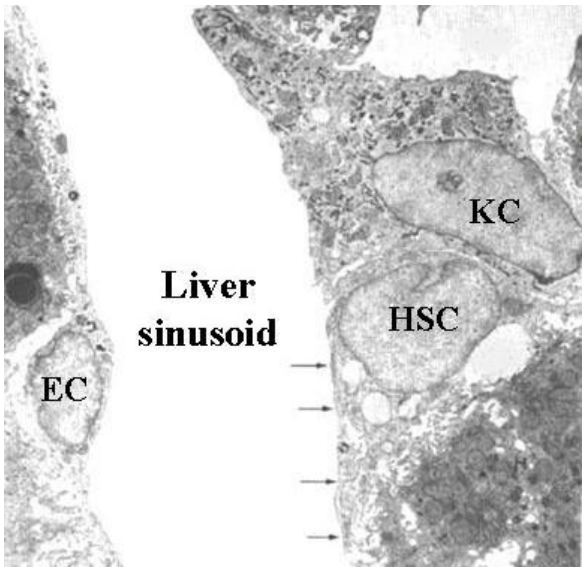

Fig.1.1. Liver sinusoid

(Arias *et al.*, 1982)

Table 1.1. Types of non-parenchymal liver cells

(Decker, 1987; Blouin *et al.*, 1977)

		Sinusoidal cell	
		Volume	Number
	Kupffer cells (KC)	44%	47,5%
	Hepatic Stellate cells (HSC)	33%	37,5%
	Endothelial cells (EC)	22%	12,5%
	Pit cells	1%	2,5%

A variety of functions has been reported for sinusoidal cells, like: filtration of particles (EC), endocytosis (EC, KC), phagocytosis (KC), secretion of bioactive lipids (eicosanoids) (EC, KC, HSC), secretion of cytokines (EC, KC, HSC), cytotoxic activity (KC), collagen production and synthesis of proteins of extracellular matrix (ECM) during inflammation (HSC); vitamin A storage (HSC), etc. (Bouwens *et al.*, 1992; Decker, 1987). They also participate in liver growth, affecting hepatocyte's proliferation (Malik *et al.*, 2002).

1.1.2. Kupffer cells under normal physiological conditions and during inflammation

Kupffer cells – resident liver macrophages – play a critical role in the maintenance of normal liver function and in the defence of the liver under inflammatory conditions (Bouwens *et al.,* 1992; Arias *et al.*, 1982; Decker, 1987).

Liver macrophages represent the largest population of tissue macrophages and constitute about 80-90% of macrophages of reticuloendothelial system (RES) (Biozzi *et al.*, 1965). Within the liver they have a strategic anatomic position to control substances coming from the intestinal tract (Bouwens *et al.*, 1992). Under physiologic conditions Kupffer cells are the long-living and self-renewing macrophage's population, whose kinetics differ from other sinusoidal cells. Three immunocytohemically and functionally different populations of Kupffer cells have been found in normal rat liver. They are situated in the different area of liver lobulus (Table 1.2.).

Kupffer cells can be distinguished from other sinusoidal cells by microscopy, histochemical analysis (endogenous peroxidase and tartrate-resistant acid phosphatase staining) and phagocytosis (Arias *et al.*, 1982).

Table 1.2. Zonal distribution and characteristics of Kupffer cells

	Zone of lobulus		
	periportal *Zone 1*	midzonal *Zone 2*	perivenous *Zone 3*
Distribution of KC, % [1), 2)]	43[1)] / 44[2)]	28[1)] / 33[2)]	29[1)] / 22[2)]
Cell size [1), 3)]	"Large"		"Small"
Characteristic [3)]	Mononuclear phagocyte		Small MC/MP/KC
ED1/ED2 [3)]	ED1+ ED2+		ED1+ ED2-
Phagocytosis [1), 3), 4), 5)]	+++		++ [1), 3), 4)]/+++ [5)]
Lysosomal enzymes [1), 5)]	++		+
Galactosyl receptors [1), 5)]	+		++
Cytotoxicity [1), 5)]	+		++
LPS-induced IL-1 synthesis	+		+
Secretory response [4)]:			
- resident cells	++		+
- LPS-Stimulated cells	+		++

1) Bouwens *et al.*, 1986; **2)** Sleyster *et al.*, 1982; **3)** Armbrust *et al.*, 1996; **4)** Bykov *et al.*, 2004; **5)** Bouwens *et al.*, 1992

Under inflammatory conditions Kupffer cells exert a large number of functions, including phagocytic activity, various immune reactions, the uptake and catabolism of lipids and enzymes, the secretion of bioactive factors, cytotoxicity, antigen processing, lipoprotein metabolism etc. (Bouwens *et al.*, 1992; Decker, 1990; Arias *et al.*, 1982). Moreover, there is evidence, that Kupffer cells possess a large capacity to kill tumor cells, resulting in a considerable defence of liver against metastasing cells (Decker, 1987). Additionally, during liver regeneration Kupffer cells play a stimulatory role by enhancing hepatocyte growth factor (HGF) expression (Takeishi *et al.*, 1999).

1.1.3. Liver fibrosis

Hepatic fibrosis is a wound repairing process, defined by the accumulation of extracellular matrix proteins, especially collagen I and III, and by the synthesis of fibronectin, proteoglycan and laminin during injury as a response on chemical and biological stress (Kershenobich *et al.*, 2003; Johnson, 1996). The major source of these compounds are the Stellate cells, which are thought therefore to be responsible for hepatic fibrogenesis and development of cirrhosis (Sokol, 2002).

Liver injury often results in an activation of Kupffer cells, involving an increased phagocytosis and secretion of a large number of mediators, which, in turn, leads to an activation of T cells, hepatic Stellate and endothelial cells, magnification of the inflammatory response and stimulation of fibrogenesis (Sokol, 2002).

The activated HSC undergo transformation into myofibroblasts with enhanced formation of ECM and synthesize a number of secretory products, including TGF-β, endothelin, matrix metalloproteases etc. (Albanis *et al.*, 2001). Sinusoidal endothelial cells express intracellular adhesion molecule 1 leading to adhesion of neutrophils and magnification of inflammatory response. Furthermore, they enhance fibrosis through the modulation of plasmin-generating enzymes in response to transforming growth factor-beta 1 (Rieder *et al.*, 1993). Defenestration and capillarization of the sinusoidal endothelium leads to a development of the fibrotic process (Braet *et al.*, 2002).

1.2. Kupffer cell derived mediators

Liver macrophages are the major producers of mediators during the inflammation (Table 1.3.) After their participation in the development of liver inflammation and fibrosis, these secretory products of Kupffer cells can be divided into two groups : (pro)-fibrogenic mediators such as IL-1β, TNFα, , IL-6, TxA_2, PDGF, TGF-β (Kawada N., 1999; Malik *et al.*, 2002; Tsukamoto, 1999; Davis *et al.*, 1996; Katagiri *et al.*, 2004; Fisher *et al.*, 1987) and anti-fibrogenic agents like PGE_2, IL-10, NO (Dieter, 1996; Davis, 1997; Kawada *et al.*, 1992).

Table 1.3. Basic biological active substances of liver macrophages

Lipids	Peptides	Inorganical substances
Prostanoids:	**Cytokines:**	
PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, TxA_2 [1), 2)]	IL-1, IL-6, IL-10, TNFα [1), 2)]	Reactive oxygen intermediates [1), 2)]
Leukotrienes:	IL-12, IL-18 [3)]	Nitric oxide [1), 2)]
5-HETE, LTA_4	IL-8 [4)]	Carbon monoxide [8)]
	IFNα/β [1), 2)]	
PAF [1), 2)]	**TGF-β** [5)]	
	PDGF	
	ET-1 [6)]	
	MIPs, MCP, KC/GRO [7)]	

1.2.1. Prostanoids

Prostanoids are potent lipid eicosanoid mediators derived from arachidonic acid (Marks *et al.*, 1999). Within the liver, Kupffer cells are the major producers of prostanoids. They release prostaglandin E_2, D_2, $F_{2\alpha}$ and thromboxane A_2 (Table 1.4.).
In opposite, hepatocytes appear to degrade prostanoids (Tran-Thi *et al.*, 1987).

1.2.1.1. Functions of prostanoids

The functions of prostanoids are listed in the Table 1.5.

---

1) Decker, 1990; **2)** Dieter, 1996; **3)** Tsutsui *et al.*, 1997; **4)** Hisama *et al.*, 1995; **5)** Decker, 1991; **6)** Treffkorn *et al.*, 2004; **7)** Bautista, 2000; **8)** Goda *et al.*, 1998

Table 1.4. Distribution of prostanoid production among liver cells (Dieter *et al.*, 2001)

Liver cells	Prostanoid formation, %				
	Total	Profile			
		PGD_2	**PGE_2**	**$PGF_{2\alpha}$**	**TxA_2**
HC	-	-	-	-	-
EC	35	12,25	6,30	5,25	11,20
KC	60	45,00	4,80	1,20	9,00
SC	4	1,48	0,88	0,16	1,48
Other liver cells	1	-	-	-	-

Table 1.5. Functions of prostanoids

General functions	*In the liver*
Prostanoids	
▪ Regulation tumor growth [1)] ▪ Modulation of different (patho)physiological processes (inflammation, immune regulation, arthritis, cancer etc.)	▪ Up-reg. glycogenolysis in perfused liver after zymosan [2)] ▪ Up-/Down-reg. Ca^{2+} and K^+ homeostasis [2)] ▪ Up-/Down-reg. contraction of HSC [2)] ▪ Regulate the size of fenestrae [3)]
PGE_2	
▪ Modulation of immune response [4)] ▪ Pain response [1)] ▪ Fever generation [5)] ▪ Bone metabolism [5), 6)] ▪ Fertilization [5)] ▪ Causes vasodilatation [4)] ▪ Chemotaxis [4)] ▪ Down-reg. release of AA, formation of IP and DAG [9)] ▪ Tumorigenesis [6)] ▪ Alzheimer disease [6)]	▪ Regulates delivery of blood substances to parenchymal cells [6)] ▪ Up-reg. portal pressure [8)] ▪ Up-reg. glycose homeostasis [2), 7)] ▪ Down-reg. O_2 uptake [8)] ▪ Down-reg. hepatic blood flow [8)] ▪ Down-reg. hepatic hemodynamics [8)] <u>In hepatocytes:</u> ▪ Up-reg. glycogenolysis [2)] ▪ Up-reg. hepatocyte's DNA synthesis [10)] ▪ Down-reg. cAMP production [11)] ▪ Down-reg. the ammonium uptake and urea formation [8)] <u>In Kupffer cells:</u> ▪ Mediates the release of inflammatory mediators [12)] ▪ Up-reg. cAMP production [11)] ▪ Down-reg. release of AA, formation of IP and DAG [2)] ▪ Down-reg. LPS-induced release of TNFα, IL-1, IL-6 [14)] ▪ Affects the LPS-induced release of NO [2), 15), 16)] ▪ Plays an autoregulatory function in KC [11)] <u>In endothelial cells:</u> ▪ Regulates the size of fenestrae [3)] ▪ Regulates the exchange of material between the blood and liver parenchyma [17)]

Continued on next page

Table 1.5. (continued from page 6)

General functions	*In the liver*
$PGF_{2\alpha}$	
▪ Up-reg. blood pressure [18)] ▪ Contraction of bronchial, vascular and arterial smooth muscle [19)] ▪ Down-reg. platelet aggregation [18)] ▪ Natriuresis [18)]	▪ Contraction of HSC, that may regulate the blood flow in the liver [20)] ▪ Down-reg. bile flow and bile acid secretion [21)] ▪ Regulates glucose metabolism [22)] ▪ Regulates blood flow and exchange of substances between the blood vessels and the hepatocytes [20)] ▪ Up-reg. hepatocyte's DNA synthesis [10)] ▪ Mediator of intercellular communication between PC and NPCs [23)]
PGD_2	
▪ Neuromodulator: promotes sleep [24)] and hypothermia [27)] ▪ Inhibition of platelet aggregation [26), 27)] ▪ Relaxation of smooth muscle contraction [26), 27)] ▪ Induction of bronchoconstriction [26), 27)] ▪ Attraction of inflammatory cells (T2 cells, eosinophils and basophils) [26), 27)]	
TxA_2	
▪ Promotion of platelet activation [28)] ▪ Contraction of vascular smooth cells and glomerular mesangial cells [29)] ▪ Mediation in thrombotic, vasospatic and bronchospatic conditions [30)] ▪ Modulation of blood flow distribution and airway calibre [30)]	▪ Potent vasoconstrictor in the intrahepatic circulation system [31)] ▪ Up-reg. glycogenolysis in perfused liver [31)] ▪ Down-reg. bile flow and bile acid secretion [21)] ▪ Up-reg. portal resistance during liver injury [32)] ▪ Contraction of HSC (that may regulate blood flow) [20)] ▪ Participates in liver injury during endotoxemia [11)] ▪ Is involved in pathogenesis of hepatic and endothelial dysfunction [33), 34)]

1) Bishop-Bailey *et al.*, 2002
2) Dieter *et al.*, 1987
3) Gatmaitan *et al.*, 1996
4) Tilley *et al.*, 2001
5) Funk, 2001
6) Murakami *et al.*, 2004
7) Wisse *et al.*, 1999
8) Mitkov, 1990
9) Lemieux *et al.*, 2002
10) Refsnes *et al.*, 1994
11) Decker, 1990
12) Dieter *et al.*, 1999
13) Karck *et al.*, 1988
14) Goss *et al.*, 1993
15) Harbrecht *et al.*, 1995
16) Gaillard *et al.*, 1991
17) Dieter *et al.*, 2001
18) Liston *et al.*, 1985
19) Seibert *et al.*, 1987
20) Kawada *et al.*, 1992
21) Beckh *et al.*, 1994
22) Gomez-Foix *et al.*, 1989
23) Suzuki-Yamamoto *et al.*, 1999b
24) Matsumura *et al.*, 1991
25) Ueno *et al.*, 1982
26) Hirai *et al.*, 2001
27) Kanaoka *et al.*, 2003
28) Hamberg *et al.*, 1975
29) Mene *et al.*, 1986
30) Ogletree, 1987
31) Fisher *et al.*, 1987
32) Yokoyama *et al.*, 2003
33) Katagiri *et al.*, 2004
34) Graupera *et al.*, 2003

1.2.1.2. Arachidonic acid cascade

The biosynthesis of prostanoids is composed of three successive steps, the so-called "Arachidonic acid cascade" (Fig. 1.2.): the release of AA from phospholipids by phospholipase (Ambs *et al.*, 1995; Marks *et al.*, 1999) followed by conversion of free AA into PGG_2/H_2 by PGH_2/G_2-synthases (or cyclooxygenases (COX), and the subsequent conversion of PGH_2 into different prostaglandins or thromboxane via specific final synthases (Smith *et al.*, 2000).

In liver macrophages there are two different mechanisms for the activation of AA cascade. The immediate pathway of the AA cascade is activated rapidly (within minutes) after stimuli like AA, zymosan, C3a, phorbol esters or calcium ionophores and is the result of activation of pre-existing enzymes such as $cPLA_2$, COX-1 and TxA_2 synthases. The delayed pathway shows a lag phase of several hours and is activated by e.g. LPS- and cytokine which results in the expression new enzymes such as COX-2 (Dieter *et al.*, 2000).

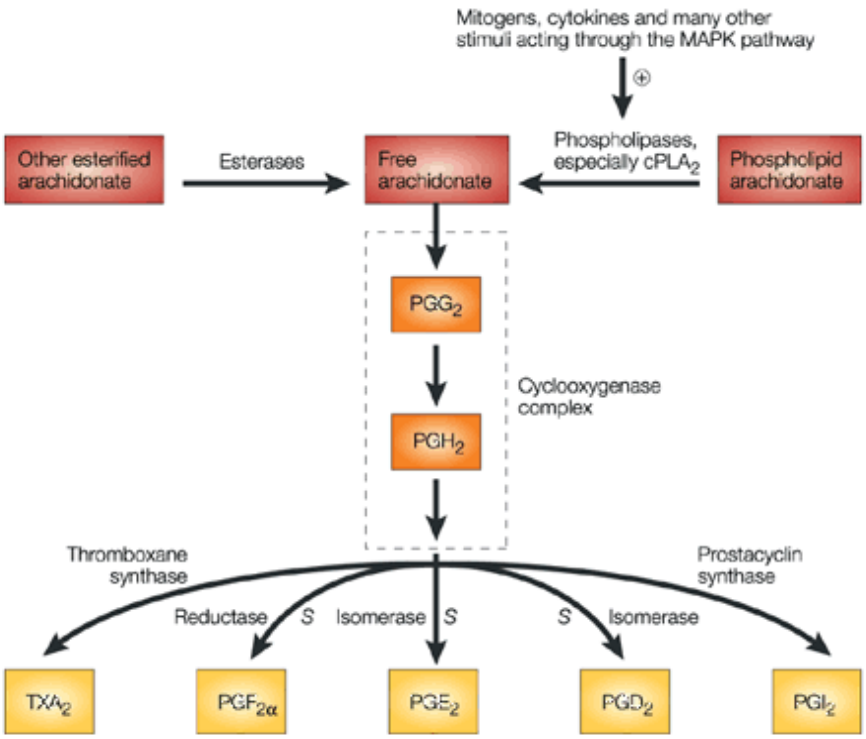

Fig.1.2. Schematic presentation of arachidonic acid cascade
(Flower et al., 2003)

1.2.1.3. Role of cPLA$_2$

The level of prostanoid synthesis is mainly defined by the availability of free AA. Under physiological conditions the intracellular level of AA is very low; it is etherified with sn-glycerol-2 position of phospholipids. The release of AA from phospholipids can occur through the action of a number of different phospholipases (PL A_2, C, D), phosphatidic acid phosphohydrolase and diacylglycerol lipase (Marks *et al.*, 1999; Diaz *et al.*, 2003; Dennis, 2000).

The (re)-incorporation of free AA (including from dietary fatty acid) into membrane phospholipids is performed through both, arachidonyl coenzyme A synthetase and lysophosphatide acyltransferase. Therefore, free AA level inside cells is dependent on the rate of its incorporation into membrane lipids versus its liberation from this pool (Fig. 1.3.) (Marks *et al.*, 1999).

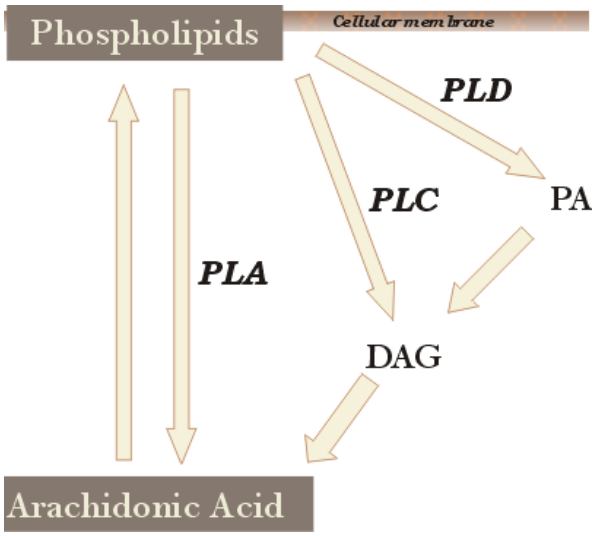

Fig.1.3. Regulation of free arachidonic acid pool
(Marks *et al.*, 1999)

The release of AA can be carried out:

1) directly, by PLA$_2$: Among a number of phospholipases A_2 groups (Marks *et al.*, 1999; Diaz *et al.*, 2003), only Group IV cytosolic phospholipase A_2 is thought to participate in the free AA generation in rat liver macrophages. Besides mRNA and proteins of Group IV cPLA$_2$

(Ambs *et al.*, 1995; Dieter *et al.*, 2002), only mRNA encoding Groups V and IIa secretory PLA_2 are constitutively expressed in liver macrophages. Cytosolic PLA_2 is defined in many cells and tissues (macrophages, platelets, neutrophils, eosinophils, endothelial cells, smooth muscle cells, alveolar epithelial cells, renal mesangial cells, keratinocytes, fibroblasts, U937, THP-1, osteoblasts, kidney, spleen, brain, lung, heart, testis) (Marks *et al.*, 1999). The protein possess 749-amino acids and has a calculated molecular weight of 85 kDa but shows a 110 kDa weight on SDS-PAGE (Ambs *et al.*, 1995). The activity of the enzyme depends on Ca^{2+} and phosphorylation (Ambs *et al.*, 1995; Dieter *et al.*, 1988).

However, for the activation of AA cascade, Ca^{2+} seems to be more important than phosphorylation (Ambs *et al.*, 1995). Ca^{2+} binds to the C2 domain of $cPLA_2$ and induces its translocation from cytosol to membranes, regulating $cPLA_2$-membrane association (Leslie, 2004).

The treatment of cells with PMA, zymosan or A23187 (Ca^{2+}-ionophor) induces a rapid upregulation of cellular free AA followed by an immediate prostanoid release (Table 1.6.) (Ambs *et al.*, 1995; Duyster *et al.*, 1992). In contrast, LPS induces an upregulation of free AA after 4h (delayed release). Recent findings (Dieter *et al.*, 2002) show that LPS induces an upregulation of $cPLA_2$-protein.

Table 1.6. Activation of $cPLA_2$
(Ambs *et al.*, 1995; Dieter *et al.*, 2004)

Agent	Effect		
	Phosphorylation of $cPLA_2$	Free Ca^{2+}	AA release
PMA	**Up-reg.** , through PKC-β and MAPK	---	**Up-reg.** release of AA (within minutes)
Zymosan	**Up-reg.** , through PKC-β and MAPK	**Up-reg.**	- `` -
A23187	---	**Up-reg.**	- `` -
LPS	**Up-reg.** , through MAPK	Up-reg.	**Up-reg.** release of AA (>4h)

2) by the combined action of phospholipase and DAG lipase: free arachidonic acid can also be generated by combined action of PLC or PLD with DAG lipase (Fig. 1.3.) (Duyster *et al.*, 1992; Dieter *et al.*, 1993).

1.2.1.4. Role of cyclooxygenases in prostanoid formation

Prostaglandin endoperoxide H synthase (or cyclooxygenase) catalyses the first two steps in the biosynthesis of prostanoids from free AA: i) Oxidation of AA to hydroperoxy endoperoxide PGG_2 (cyclooxygenase activity of enzyme) and ii) its reduction to the hydroxy endoperoxide PGH_2 (peroxidase activity) (Smith *et al.*, 2000; Vane *et al.*, 1998). Since 1990 two isoforms of COX are known – COX-1 and –2 (Table 1.7.). COX-1 is a constitutively expressed enzyme and defined in most tissues. COX-2 is an inducible enzyme and appears after the stimulation of cells by various agents, e.g. LPS, cytokines, PDGF etc. (Smith *et al.*, 2000).

Recent evidence demonstrates the existence of a splice variant of COX-1 (Chandrasekharan *et al.*, 2002), the so-called "COX-3", in canines. The expression of "COX-3" mRNA in rat central nervous system has been recently discovered (Kis *et al.*, 2004). Moreover, the existence of other COX's isoenzymes is assumed (Davies *et al.*, 2004).

Table 1.7. Characteristics of COX isoenzymes
(Marks *et al.*, 1999; Smith *et al.*, 2000***)***

	COX-1	**COX-2**
N of Amino acids	576	587
Calculated MW, kDa	66	67
Relative mobility on SDS-PAGE, kDa	72	72-74
Synthesis	Constitutive	Inducible
Main physiological functions	Regulation in stomach and intestine function	Inflammation and injury
Coupling to PLA_2	Depends on the cell type and the stimulus (Marks *et al.*, 1999, p. 77)	

1.2.1.5. Conversion of PGH_2/PGG_2 into prostanoids

The enzymatic conversion of PGH_2 into prostanoids is performed by final prostanoid synthases (Fig. 1.2.). Till now several (iso)enzymes have been isolated, characterized and cloned (Ogorochi *et al.*, 1987; Tanaka *et al.*, 1987; Meyer *et al.*, 1996; Jakobsson *et al.*, 1999b; Murakami *et al.*, 2000; Tanioka *et al.*, 2000; Tanikawa *et al.*, 2002; Lazarus *et al.*, 2002a). Since Kupffer cells synthesize PGE_2, PGD_2, $PGF_{2\alpha}$ and TxA_2, the enzymes for their production will be discussed below.

1.2.1.5a. PGE_2-synthase

The isomerization of PGH_2 into PGE_2 is catalysed by PGE_2-synthase (PGES, EC 5.3.99.3, systematic name (5Z,13E)-(15S)-9α,11α-epidioxy-15-hydroxyprosta-5,13-dienoate E-isomerase). PGES is synthesized almost in all tissues and cells, and the existence of various types of PGE_2-synthases has been proposed earlier (Ogorochi *et al.*, 1987; Tanaka *et al.*, 1987).

At present, three distinct types of PGES are identified, cloned and investigated, i.e. GSH-specific and non-specific membrane-associated synthases (mPGES-1,-2) and cytosolic type (cPGES), their properties are briefly shown in Table 1.8.

Table 1.8. Characteristics of PGE_2-synthases

	mPGES-1 [1)]	**mPGES-2** [2)]	**cPGES** [3)]
N of Amino acids	152-153	377-384	160 [4)]
Calculated MW, kDa	16-18	33	23
Relative mobility on SDS-PAGE, kDa	16	33	23-25
GSH-dependent activity	+	---	+

The first microsomal types of PGH-PGE isomerases have been partially purified from microsomal fractions of bovine (Jakobsson *et al.*, 1999a) and sheep (Moonen *et al.*, 1982) vesicular glands; the enzymes activity is dependent on glutathione (Table 1.9.).

Later, PGES has been purified and identified as a member of MAPEG protein superfamily. This group of enzymes consists of membrane associated proteins, which are involved in eicosanoid and glutathione metabolism. The glutathione-dependent membrane-bound prostaglandin E_2 synthase was previously designated as PIG12 or MGST1-L1 and is now known as mPGES-1 (Jakobsson *et al.*, 1999a; Jakobsson *et al.*, 2000; Murakami *et al.*, 2000).

---

1) Lazarus *et al.*, 2002; Jakobsson *et al.*, 1999;
2) Tanikawa *et al.*, 2002; Hu *et al.*, 2002; Watanabe *et al.*, 1999;
3) Tanioka *et al.*, 2000
4) Zhang *et al.*, 2003

Table 1.9. PGE_2-synthases: the history of investigation

	Cells, tissue	Fractions	PGH_2 activity		min GSH for max activity, mM	GSH: K_{max}, µM	pH optim	*Rel. mob. on SDS-PAGE, kDa*
			K_m, µM	V_{max}, nmol/ min/mg				
Microsomal fractions	Sheep vesicular glands[1)]	Microsomal		30	0.5		5.5-7.0	60, 67
	Sheep vesicular glands[2)]	IgG_1 (hei-7)	40		2		6,5-7,5	17,5
		IgG_2 (hei-26)	150		<0,5		6.2	180
	Rat tissues[3)]	Deferent duct etc.: GSH-dependent	25.7	≤ 112	0.2		6-7	
		Heart, spleen, uterus : GSH-independent	82.6	≤1,1	---		6-8 (9-12)	
	Bovine hearts[4)]	Microsom. fr.	24		-	-	>6.0	31
Cytosolic fractions	Numan brain cytosol[5)]	peak 1	147	380	5	61	5.4	24.5, 25
		peak 2	308	720		86	5.4	25
	Ascaridia galli[6)]				0.51	75		24.3-25.7 (7 subunits)
	Human brain [7)]	Cytosolic fr.		1.21				27.5
	Rec. (E. coli) [7)]	GST M2-2	141	282	2,5	121	8.0	26.5
		GST M3-3	1500	923		76		27.5

The cDNAs for human, mouse and rat mPGES-1 have been further cloned and their properties were investigated (Lazarus *et al.*, 2002a; Yamagata *et al.*, 2001; Jakobsson *et al.*, 1999a) (Table 1.10.). mRNA has been found at high levels in A549, HeLA cell lines, at intermediate levels in placenta, prostate, testis, mammary gland and bladder (Jakobsson *et al.*, 1999a).

1) Moonen *et al.*, 1982
2) Tanaka *et al.*, 1987
3) Watanabe *et al.*, 1997
4) Watanabe *et al.*, 1999
5) Ogorochi *et al.*, 1987
6) Meyer *et al.*, 1996
7) Beuckmann *et al.*, 2000

Table 1.10. Cloning of PGES

	Ref	Protein, host, nomenclature	Bacterial system		PGH_2 activity		GSH: K_{max}, µM	*Rel.mob. on SDS-PAGE, kDa*
					K_m, µM	V_{max}, nmol/ min/mg		
mPGES-1	1)	Rec., human, NP_004869, O14684	E. coli			250	+	15-16
	2)	Rat/mouse/human, NP_071860, NP_067594, Q9JM51, BA96084	HEK293 cells	Rat/ Mouse/ Human		≈260-370*	+	
	3)	Rec., rat, NP_067594, AAG24803	Chinese hamster ovary cells (CHO-K1)			1000	+	17
	4)	Rec., rat, NP_067594, BAB20597	Rat testis				+	
	5)	Bovine,NP_776868, AAK51127	Ovarian follicias				+	17
	6)	Rec., mouse, BAB71813, NP_071860, Q9JM51	E. coli		130	1816	37	17
	7)	Rec., human, NP_004869, O14684	E. coli		160	170* 10^3	710	18
	8)	Rec., human, O14684	Bacilovirus-Sf9		14	400	750	17
mPGES-2	9)	Rec., monkey, NP_079348, BAB01608	E. coli (BL21)		28	3300	---	33
cPGES	10)	Rec., human/Rat brain cytosol	E.coli		14	190	+	26

* ng/30 sec/10µg

Another PGES was purified from cytosol fractions of human brain (Ogorochi *et al.*, 1987) and Ascaridia galli (Meyer *et al.*, 1996) (so-called p23). Recently, the cDNA for cPGES/p23 has been identified and cloned; the protein, expressed in E.coli, exhibits GSH-requiring PGE synthase activity (Tanioka *et al.*, 2000).

Finally, the novel-type PGES has been purified from microsomal fraction of bovine heart (Watanabe *et al.*, 1999).

1) Jakobsson *et al.*, 1999b
2) Murakami *et al.*, 2000
3) Mancini *et al.*, 2001
4) Yamagata *et al.*, 2001
5) Filion *et al.*, 2001
6) Lazarus *et al.*, 2002a
7) Thoren *et al.*, 2003
8) Ouellet *et al.*, 2002
9) Tanikawa *et al.*, 2002
10) Tanioka *et al.*, 2000

This enzyme, called mPGES-2 has been identified and cloned; the examination of enzymatic properties of mPGES-2 shows GSH-independency (Tanikawa *et al.*, 2002). It requires SH reagent (especially DTT), and has the highest k_{cat}/K_m and V_{max} for PGH_2 among other PGES types (Tanikawa *et al.*, 2002). The amino acid sequence of mPGES-2 does not exhibit homology with the mPGES-1, assuming that mPGES-2 does not belong to the MAPEG protein family (Jakobsson *et al.*, 1999a; Tanikawa *et al.*, 2002; Jakobsson *et al.*, 2000). The mPGES-2 mRNA is detected in many cells and tissues (genital organ, brain, lymph nodes, heart, skeletal muscle, kidney and trachea) (Tanikawa *et al.*, 2002).

1.2.1.5b. PGD_2-synthase

Prostaglandin D_2 is synthesized by PGD_2-synthase (Christ-Hazelhof *et al.*, 1982) (PGDS, EC 5.3.99.2, (5,13)-(15S)-9α,11α-epidioxy-15-hydroxyprosta-5,13-dienoate D-isomerase). The function of PGDS in the liver is not get clear; the short characteristic for PGDS is provided above (Table 1.5.) (the more detailed information see reviews: Marks *et al.*, 1999; Urade *et al.*, 2002; Kanaoka *et al.*, 2003).

Two isoforms of enzyme have been purified and further investigated:

1) Lipocain PGDS ("brain type"): GSH-independent secretory enzyme from lipocain superfamily. It has been isolated firstly from rat brain (Shimizu *et al.*, 1979); it is expressed in the brain and testis and secreted into cerebrospinal fluid and the seminal plasma as β-trace (Tanaka *et al.*, 1997).
2) Hematopoietic PGDS ("spleen type"): it is GSH-dependent, cytosolic enzyme, has been isolated firstly from rat spleen (Christ-Hazelhof *et al.*, 1982) and is expressed by mast cells, histiocytes and dendritic cells of the spleen, thymus, skin and KC in the liver (Kanaoka *et al.*, 2003).

Both isoenzymes convert PGH_2 into PGD_2, consist of 183-199 amino acid and have the molecular weight 21-27 kDa (Urade *et al.*, 1989; White *et al.*, 1992; Hoffmann *et al.*, 1993).

1.2.1.5c. $PGF_{2\alpha}$-synthase

Prostaglandin $F_{2\alpha}$ is secreted *in vivo* in form of 9α-,11α-PGF_2, i.e. $PGF_{2\alpha}$ (which is also synthesized by Kupffer cells) and 9α,11β-PGF_2, i.e. 11-epi $PGF_{2\alpha}$ (Marks *et al.*, 1999). Three pathways exist for prostaglandin $F_{2\alpha}$ synthesis (Watanabe, 2002; Helliwell *et al.*, 2004):

1) from PGH_2 to $PGF_{2\alpha}$: PGH_2 9,11-endoperoxide reductase-activity of $PGF_{2\alpha}$-synthase (PGFS);
2) from PGE_2 to $PGF_{2\alpha}$: PGE_2 9-ketoreductase-activity of PGFS;
3) from PGD_2 to 11-epi $PGF_{2\alpha}$: PGD_2 11-ketoreductase-activity of PGFS.

$PGF_{2\alpha}$-synthases are all NAPDH-dependent and have 323 amino acids / MW about 36-37. They are classified by both origins and enzymatic properties:

1) $PGF_{2\alpha}$-synthase (EC 1.1.1.188): this enzyme shows 11-ketoreductase-activity and 9,11-endoperoxide reductase-activity.

Two isoenzymes exist:

- **Lung-type PGFS I**: was isolated firstly from bovine lung (Watanabe *et al.*, 1985); $K_m(PGH_2)$=10 μM; $K_m(PGD_2)$=120 μM;
- **Liver-type PGFS II**: was isolated firstly from bovine liver (Chen *et al.*, 1992); $K_m(PGH_2)$=25 μM; $K_m(PGD_2)$=10 μM;

The difference between the isoforms are: different sensitivity of $PGF_{2\alpha}$, to metals, chloride ions, salts ($CuSO_4$, $HgCl_2$) and immunoprecipitation (Chen *et al.*, 1992). Both isoenzymes were characterized, cloned and expressed in E. coli (Watanabe *et al.*, 1991; Suzuki *et al.*, 1999; Suzuki-Yamamoto *et al.*, 1999a).

2) Aldehyde reductase (EC 1.1.12) was isolated firstly from human liver (Hayashi *et al.*, 1989). It shows only PGH_2 9,11-endoperoxide reductase-activity, $K_m(PGH_2)$=100 μM; PGD_2-activity was not detected.

1.2.1.5d. TxA_2-synthase

Thromboxane A_2 is synthesized by TxA_2-synthase (TxAS, EC 5.3.99.5, (5Z,13E)-(15S)-9a,11a-epidioxy-15-hydroxyprosta-5,13-dienoate isomerase). It has been firstly identified in the microsomal fraction of platelets (Needleman *et al.*, 1976), thereafter in rat seminal vesicles (Moncada *et al.*, 1976) (Table 1.11.).

Table 1.11. TxA_2 synthase: history of their investigation

Cells, tissue	Ref.	PGH_2 activity		pH optim	Ratio TxA_2:HHT	*Relative mobility on SDS-PAGE, kDa*
		K_m, μM	V_{max}, nmol/ min/mg			
Human/horse platelets	1)					
Rat seminal vesicles	2)					
Human platelets	3)					
Human platelets	4)		84*	6.5-9.0		
Human platelets	5)		57		1:1	58,8
Porcine lung	6)	12		7.5	1:1,6-2.0	53
Human platelets	7)	24				
Human paltelets	8)	10			1:1	58

*nmol/20sec/mg

Thromboxane synthase consists of 533-534 amino acids and has a molecular weight of 58-60 kDa (relative mobility 56-60) dependent on the host organism (Wang *et al*., 2002; Shen *et al.*, 1998).

TxA_2 synthase is a membrane cytochrome $\boldsymbol{P}_{450}$-like protein: UV-VIS, MCD and EPR indicate (Hsu *et al.*, 1999) that TxAS has a typically low spin cytochrome P450 heme with an oxygen-based distal ligand (Haurand *et al.*, 1985).

TxAS catalyzes two reactions with the same substrate - PGH_2: 1) the conversion of PGH_2 into TxA_2 and 2) the cleavage of PGH_2 to form 12-HHT and malondialdehyde (Hecker *et al.*, 1989). Enzymes, purified from platelets, and recombinant kidney TxAS produced equal amounts of TxA_2 and 12-HHT (Hsu *et al.*, 1999). Enzymes, purified from porcine lung, and for recombinant protein from baculovirus system, the ratio of synthesized TxA_2:12-HHT was approximately 1:2 (Yokoyama *et al.*, 1993). During catalysis, TxAS undergoes a "suicide" inactivation, which occurs at the reactive site of the enzyme (Jones *et al.*, 1990).

1) Needleman *et al.*, 1976;
2) Moncada *et al.*, 1976;
3) Sun, 1977;
4) Hammarstrom *et al.*, 1977;
5) Haurand *et al.*, 1985;
6) Shen *et al.*, 1986;
7) Jones *et al.*, 1990;
8) Nusing *et al.*, 1990.

DNA of TxAS has been cloned (Table 1.12.), and the recombinant protein (*E. coli*) showed a high specific activity, as purified enzyme (Hsu *et al.*, 1999; Yokoyama *et al.*, 1993).

The existence of two isoforms of TxAS in human lung has been supposed after screening of human lung cDNA library (Ohashi *et al.*, 1992), but last publications reported about only one known isoforms (Wang *et al.*, 2002).

Table 1.12. Cloning of TxAS

Protein, host, nomenclature	**Tissue, cells/Bacterial system**	**Ref.**	**PGH_2 activity**		**Ratio TxA_2:HHT :MDA**	***Relative mobility on SDS-PAGE, kDa***
			K_m, µM	**V_{max}, nmol/ min/mg**		
Human platelet, NP_112246, NP_001052; BAA07011, S48161, A41766	Spodoptera frugiperda Sf9	1)		130	**1.0:1.8**	58
Human lung, NP_001052, P24557, A41766, AAA60618		2)	TxAS I			534aa/60,6*
Human lung, NP_112246, B41766, AAA60617			TxAS II			460aa/52,4*
Mouse, lung, NP_035669, AAB39850, P36423, JN0683		3)				
Rat, bone-marrow cells, NP_036819, P49430, BA05962, S42404	Peritoneal macrophages	4)				
Porcine lung, NP_999211, P47787, AA31127	Spodoptera frugiperda Sf9	5)				60,5*
Rat kidney, NP_036819, P49430, BAA22574		6)				
Recomb., human	E. coli	7)	20	12000	**0.94:1.0:0.93**	56

* *calculated MW*

1) Yokoyama *et al.*, 1991; Yokoyama *et al.*, 1993
2) Ohashi *et al.*, 1992
3) Zhang *et al.*, 1993
4) Tone *et al.*, 1994
5) Shen *et al.*, 1994
6) Tsutsumi *et al.*, 1997
7) Hsu *et al.*, 1999

1.2.2. Nitric oxide

1.2.2.1. Nitric oxide and its main functions in the liver

Nitric oxide is the product of the nitric oxide synthase (NOS). It exists as a number of inorganic free radicals such as ·NO, NO^-, NO^+, NO_2, NO_2^-, NO_3^-, N_2O_3, S-nitrothiols, peroxynitrile or nytrosil-metal complexes (Bogdan, 2001). The enzymatic reaction includes the oxidation of the one guanidine nitrogens of substrate L-arginine by molecular oxygen to form NO and L-citrulline (Muriel, 2000) (Fig. 1.4.).

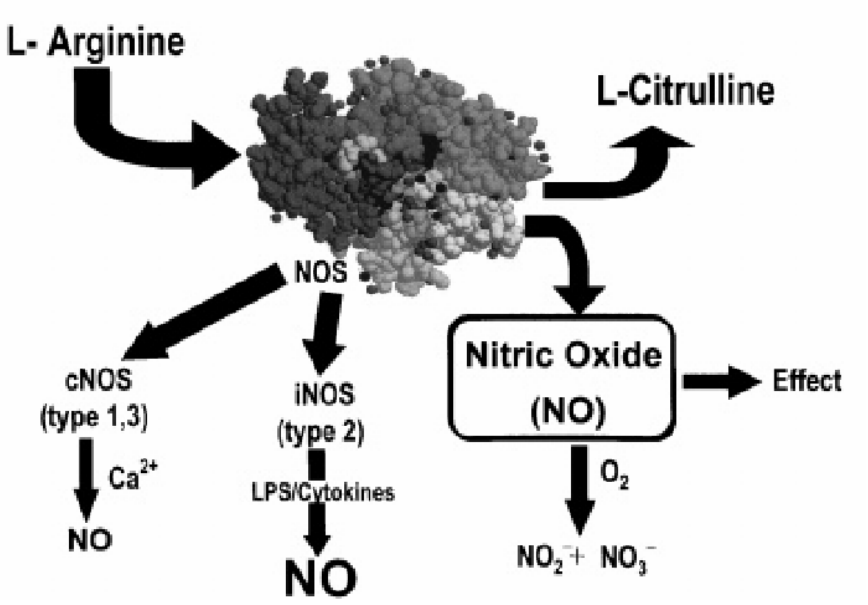

Fig.1.4. Nitric oxide synthesis from L-arginine
(Muriel *et al.*, 2000)

Nitric oxide has been identified at first as an endothelial derived relaxation factor (EDRF) (Ignarro, 1990) and antiplatelet substance. It is produced in neuronal cells (Bredt *et al.*, 1990), leukocytes (McCall *et al.*, 1989), macrophages and endothelial cells (Curran *et al.*, 1989) and other cells (Amber *et al.*, 1988; Moncada *et al.*, 1991).

The role of NO in the liver is not yet clear. Enhanced production of NO during cirrhosis might lead to the development of the hyperdynamic circulation, whereas the local deficiency of NO in the liver might lead to portal hypertension (Table 1.13.).

Table 1.13. Functions of NO in the injured liver

- exerts a hepatoprotective effect during endotoxemia (Harbrecht *et al.*, 1995; Nishida *et al.*, 1994; Wang *et al.*, 1995)
- **Down-reg.** the platelet aggregation (Mellion *et al.*, 1981)
- **Down-reg.** the contraction of SC and affects the hepatic microcirculation (Farzaneh-Far *et al.*, 2001)
- establishes of the hyperdynamuic circulation (Farzaneh-Far *et al.*, 2001)
- plays both cytotoxic and cytoprotective role during liver injury and carcinogenesis (Farzaneh-Far *et al.*, 2001; Vos *et al.*, 1997; Lirk *et al.*, 2002)
- promotes injury though its oxidant properties (Gardner *et al.*, 1998)
- inhibited of the mitochondrial respiratory chain enzymes and gluconeogenesis (Horton *et al.*, 1994)
- inhibits total protein synthesis in hepatocytes (Curran *et al.*, 1990)
- prevent necrotic cell death following endotoxemia-induced liver injury (Ou *et al.*, 1997)
- preventes hepatocytes apoptosis *in vitro* and *in vivo* (Jones *et al.*, 1998; Li *et al.*, 1999)

1.2.2.2. Synthesis of nitric oxide

NO synthases (NOS) catalyse the oxidation of L-arginine to nitric oxide and citrulline. Three subtypes have been identified:

- **neuronal NOS** (type 1, nNOS) is a constitutive isoform, Ca^{2+}/calmodium dependent, main function in neurotransmission;

- **inducible NOS** (type II, iNOS) is not expressed under normal conditions, but can be induced by cytokines and LPS in many cell types (Nussler *et al.*, 1993; Vos *et al.*, 1997), in response to liver injury from diverse insults, including hepatotoxins, endotoxemia, and ishemia-reperfusion (Nussler *et al.*, 1993; Kroncke *et al.*, 1997); the localization of iNOS is usually assumed to be cytoplasm, but the study of endotoxemia (Vos *et al.*, 1997; Nussler *et*

al., 1993) showed hepatocyte's iNOS more intensely in the plasma membrane than in the cytoplasm.

- **endothelial** (type III, eNOS) - constitutive isoform, Ca^{2+}/calmodium dependent, importent role in vasorelaxation.

NOS are primarily cytosolic, although eNOS can be mirystilated and localized in membranes (Appleton *et al.*, 1996). Both constitutive synthases rapidly synthesize small amounts of NO in response to increases in intracellular calcium and supposed to regulate physiological NO homeostasis and cellular signalling (Leifeld *et al.*, 2002). The inducible NO synthase is under transcriptional control and produces high amount of NO induced by cytokines or endotoxin**.**

The increased NO production in the liver results from enchanced expression of iNOS in hepatocytes and KC: LPS is a weak inducer of hepatocyte's iNOS; iNOS in the LPS-treated liver, highly expressed in inflammatory cells and Kupffer cells (Vos *et al.*, 1997; Pollock *et al.*, 1995; Moncada *et al.*, 1991).

1.2.2.3. Regulation of NOS

The NOS activity is also dependent on a number cofactors, including FAD, FMN; H_4B and glutathione (Moncada *et al.*, 1991; Muriel, 2000). Several NO synthases inhibitors are used in the studies of NO signalling: salycilates **(**Vos *et al.*, 1997**)**; N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monometyl-L-arginine (L-NMMA), which inhibit more specifically eNOS than iNOS (Vos *et al.*, 1997**);** S-metylisothiourea (SMT) and L-NIL (L-N^6-(1-Iminoethyl) lysine, which are preferential iNOS inhibitors **(**Vos *et al.*, 1997**)**.

1.2.3. Synthesis of cytokines by rat liver macrophages

Cytokines constitute a major class of mediators responsible for activation of liver cells *in vitro* and *in vivo*. They can be divided into pro-fibrogenic (interleukin-1, tumor necrosis factor-alpha) and anti-fibrogenic (interleukin-6, interleukin-10) and mitogenic (transforming growth factor-alpha, platelet-derived growth factor, insulin-like growth factor), mediators (Tsukamoto, 1999; Decker, 1990). Elevated amounts of TNFα and IL-1β has been found in culture medium of LPS-stimulated rat liver slices (Olinga *et al.*, 2001).

1.2.4. Influence of mediators on the development of liver fibrosis

One initial step of the hepatic inflammation is the activation of resident liver macrophages and the subsequent secretion of a large number of inflammatory mediators. The secreted mediators induce a new wave of cell interactions and responses, leading i) to activation, proliferation of liver Stellate cells and their transformation into myofibroblasts; ii) to the activation of hepatocytes and liver endothelial cells resulting in impaired blood flow and further ischemic injury; iii) to deposition of excessive extracellular matrix resulting in hepatic fibrosis (Sokol, 2002; Kershenobich *et al.*, 2003; Bissell, 1998). The prolonged hepatocellular damage may progress to cirrhosis in which the replacement of the normal hepatic constitution is replaced by fibrous septa and nodules of regenerative parenchyma (Johnson, 1996).

Taking all data together, the following scheme (Fig. 1.5.) summarizes the influence of two groups of Kupffer cell-derived products on other liver cells during the liver inflammation, accelerating (pro-fibrogenic mediators; reported by Kawada N., 1999; Malik *et al.*, 2002; Davis *et al.*, 1996; Cruz-Gervis *et al.*, 2002; Kawada *et al.*, 1992; Fisher *et al.*, 1987) or suppressing (anti-fibrogenic mediators; reported by Ju *et al.*, 2002; Dieter, 1996; Davis, 1997; Kawada *et al.*, 1992) the further development of liver inflammation and fibrogenesis.

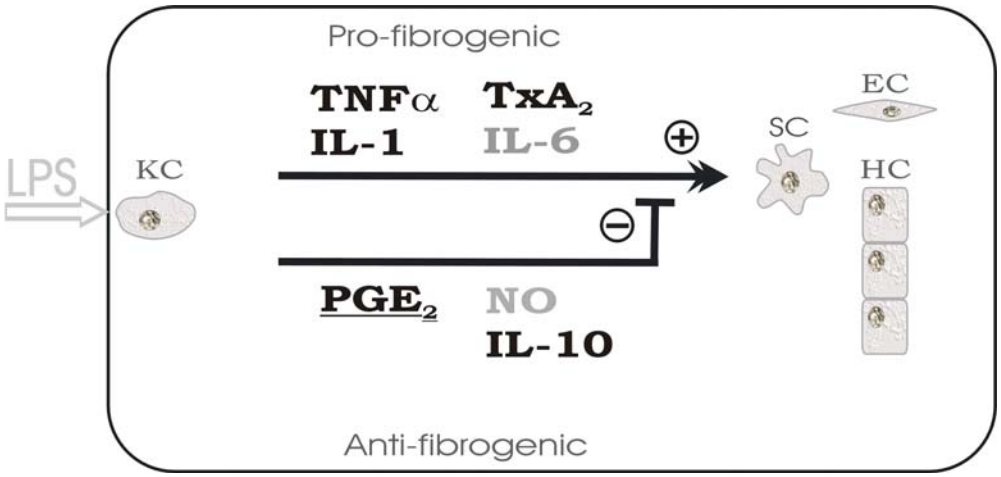

Fig. 1.5. The development of liver fibrosis is under control of pro- and anti-fibrogenic substances

1.3. Purpose of study

The present study is focused on the synthesis of inflammatory mediators (PGE_2, TxA_2, NO, interleukins) by liver macrophages under inducible conditions. The released products promote (IL-1β, TNFα, TxA_2) or abrogate (PGE_2, NO, IL-10) the liver injury. The reciprocal action between anti- and pro-inflammatory mediators operates the wound's trend. This work is therefore directed on the autocrine activation of macrophage by pro-inflammatory mediators which causes the enlarged production of protective substances and delays the further development of inflammation.

Firstly, the purpose of study is the investigation of prostanoid (PGE_2 and TxA_2) release *in vitro* upon stimulation and a long period of incubation and the characterization of enzymes involved in their synthesis. Furthermore, the coupling of $cPLA_2$, COX isoenzymes and PGE_2 synthases is characterized.

Secondly, the presented study evaluates the autocrine effect of pro-fibrogenic and anti-fibrogenic cytokines on prostanoid production.

Thirdly, the transcriptional and translational regulation of NO synthesis in liver macrophages and the interaction between prostanoids and NO is investigated.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Equipment and materials

2.1.1.1. Instruments

Automated gel analysis system „GeneGenius“	Syngene (Cambridge, UK)
Centrifuges:	
Beckman J2-21M/E high-speed centrifuge	Beckman Instruments (Munich, Germany)
Beckman Ultracentrifuge Optima TL-100	Beckman Instruments (Munich, Germany)
Eppendorf 5417 -R, -C	Eppendorf (Hamburg, Germany)
Sigma 4K10	B. Braun (Melsungen, Germany)
Color Scanner Umax PowerLook II	Umax Systems GmbH (Willich, Germany)
EHRET Biosafe ET 130/KI2	Ehret GmbH & Co. KG (Emmendingen, Germany)
Electric balance „BP2100S“	Sartorius AG (Göttingen, Germany)
Electric balance „Analytic AC120S“	Sartorius AG (Göttingen, Germany)
Electrophoresis chambers:	
Horisontal, „Agagel maxi”	Biometra (Göttingen, Germany)
Horisontal, „Mini”, “Midi”	Harnischmacher-Labortechnik (Kassel, Germany)
Verical SE600	Hoeffer Scientific Instruments (San Francisco, CA, USA)
Verical Bio-Rad Mini Cell	Bio-Rad Laboratories (Munich, Germany)
Electrophoresis power supply:	
Standard Power Pack P25	Biometra® (Göttingen, Germany)
Power Pac 3000	Bio-Rad Laboratories (Munich, Germany)
Mini Protean II	Bio-Rad Laboratories (Munich, Germany)
High performance chemiluminescence’s system “Genegnome”	Syngene (Cambridge, UK)
Gilson Pipetman Pipette	Gilson International B.V. (DEN HAAG The Netherlands)
Gradient former Model 385	Bio-Rad Laboratories (Munich, Germany)
Incubator Heraeus BS 6000	Heraeus Med GmbH (Hanau, Germany)
Laminar downflow cabinet Faster LS90 model „TWO30”	Faster Bio-Flow-Technik (Germany)
Magnetic stirrer and temperature controller Heidolph MR 2002	Heidolph (Kehlheim, Germany)
Microscopes:	
Zeiss Microscipe	Carl Zeiss (Göttingen, Germany)
Mikroscope Wilovert S	Hund GmbH (Germany)

Microwave Oven AEG Micromat	AEG (Nuernberg, Germany)
Milli-Q-Plus PF water purification system	Millipore (Eschborn, Germany)
Mini TransBlot Electrophoretic Transfer Cell	Bio-Rad (Italy)
Minishaker MS2 IKA®	IKA Works, Inc. (Wilmington, NC, USA)
Osmometer Vogel Digital, OM-802	Vogel GmbH (Giessen, Germany)
Orbital incubator "Innova 4330"	New Brunswich Scientific GmbH (Germany)
PCR thermocyclers:	
Perkin Elmer GeneAmp PCR System Model 2400	PerkinElmer Life Sciences (Boston, MA, USA)
Mastercycler®/ Mastercycler® Gradient	Eppendorf GmbH (Hamburg, Germany)
Peristaltic pump MasterFlex Digi-Staltic Model 7525-36	Cole-Parmer Instrument Company (Barrington, IL, USA)
pH-Meter Knick model 763 Multi-Calimatic	Knick Elektronische Messgerate (Berlin, Germany)
Pipetting support: Pipettus Accu®	Hirschmann Laborgeräte (Eberstadt, Germany)
Plate reader Dynatech MRX	Dynatech Laboratories Ltd. (Billingshurst, UK)
Reagent glass shaker Reax 2000	Heidolph (Germany)
Spectrophotometers:	
NanoDrop® ND-1000 UV-Vis Testimonial	G.Kisker GbR (Steinfurt, Germany)
LKB Pharmacia Ultrospec III UV/VIS	Pharmacia LKB Biochrom (Cambridge, UK)
Shimadzu UV-Mini 1240 UV-Vis	Shimadzu Deutschland GmbH (Duisburg, Germany)
Termomixer Comfort	Eppendorf (Hamburg, Germany)
Ultrasonic cleaner Bandelin Sonorex RK100	Bandelin GmbH (Berlin, Germany)
Ultrasonic homogenizer Sonopuls HD-70	Bandelin GmbH (Berlin, Germany)
Waterbath Grant Sub 6	Grant (Cambridge, UK)

2.1.1.2.Equipment

26-gauge spinal needle	B. Braun (Melsungen, Germany)
Cell scraper	Greiner Bio-One GmbH (Frickenhausen, Germany)
Centrifuge conic tubes 15 cm^2, 50 cm^2	Greiner Bio-One GmbH (Frickenhausen, Germany)
Cellulose-acetate sheets	Schleicher und Schöll (Dassel, Germany)
Injekt 40 syringe	B. Braun (Melsungen, Germany)
Neubauer cell counting chamber	Paul Marienfeld GmbH (Bad Mergentheim, Germany)
Nylon Net Filter roll hydrophilic 100 µm	Millipore (Eschborn, Germany) (#NY1H00010)
Nitrocellulose membrane Hybond™-c	Amersham Life Science (Beckingham, UK) (#RPN303E)
Parafilm	American National Can (Greenwich, CT, USA)
PCR Reaction tubes	Greiner Bio-One GmbH (Frickenhausen, Germany) #671272-7
Safe-Lock Tubes 0.5-2 ml	Eppendorf (Hamburg, Germany)
Pipette tips	Greiner Bio-One GmbH (Frickenhausen, Germany)
Tissue culture dish, Falcon 3001 Ø 35 mm	Becton Dickinson (Franklin Lakes, NJ, USA)
X-ray films	Amersham Life Science (Beckingham, UK)
96 well plate	Greiner Bio-One GmbH (Frickenhausen, Germany), Nunc (Roskilde, Denmark)

2.1.1.3. Animals

Male Wistar rats	Charles River (Sulzfeld, Germany)

2.1.1.4. Special software for molecular biology

ImageQuaNT® 5.0	Molecular Dynamics®/Amersham Biosciences
GeneSnap 3.00.25	Syngene (Cambridge, UK)
NanoDrop 2.5.4	G.Kisker GbR (Steinfurt, Germany)
Primer3	Whitehead Institute for Biomedical Research (Rozen S. *et al.*, 2000)
BLAST (NCBI)	National Center for Biotechnology Information (Bethesda, MD, USA) (Madden *et al.*, 1996; Zhang *et al.*, 2000)

2.1.2 Chemicals

All buffers and solutions are made in sterile H_2O, prepared by Milli-Q-Plus PF water purification system unless otherwise stated.

Table 2.1. Chemicals

Substances	*Company*	*Catalog No.*
A23187, free acid ($C_{29}H_{37}N_3O_6$)	Calbiochem/Merck Biosciences GmbH (Bad Soden, Germany)	#100105
ABTS	Roche Diagnostic GmbH (Mannheim, Germany)	#102946
Acetic acid	Merck KG (Darmstadt, Germany)	#1.00063.1011
Acetylsalicylic acid	Sigma (Deisenhofen, Germany)	#A-5376
Acetone	Karl Fischer Reagents	#NT 2000
Acrylamide/Bis	Bio-Rad Laboratories (Munich, Germany)	#161-0120
Agarose	Serva (Heidelberg, Germany)	#11400
Amido Black 10B	Merck KG (Darmstadt, Germany)	#1167
Ammonium persulfate	Bio-Rad Laboratories (Munich, Germany)	#161-0700
Aprotinin, 10 mM solution	Sigma (Deisenhofen, Germany)	#A-6279
Benzamidin	Sigma (Deisenhofen, Germany)	-6506
Boric acid	Merck KG (Darmstadt, Germany)	#1.00165.1000
Bromophenol Blue	Fluka AG (Buchs, Switzerland)	#18030/040
$CaCl_2$	Merck KG (Darmstadt, Germany)	#102083
Carbogen (95% O_2/5% CO_2)	Messer Griesheim (Krefeld, Germany)	
Chloroform	Sigma (Deisenhofen, Germany)	#C-5312
Citric acid	Merck KG (Darmstadt, Germany)	#241 1000
Coomassie® Brilliant Blue G250	Bio-Rad Laboratories (Munich, Germany)	#161-0400
DEPC	Sigma (Deisenhofen, Germany)	#D-5768
Dithiotheriol	Sigma (Deisenhofen, Germany)	#D-0632
DMSO	Calbiochem	#317275
dNTP's	Invitek GmbH (Berlin, Germany)	#30203012
EDCI	IRIS Biotech GmbH (Marktredwitz, Germany)	#RL-1022
EDTA	Sigma (Deisenhofen, Germany)	#E5134
EGTA	Sigma (Deisenhofen, Germany)	#E-3889
Ethanol	Merck KG (Darmstadt, Germany)	#11727

Continued on next page

Table 2.1. (continued from page 26)

Substances	*Company*	*Catalog No.*
Ethidium bromide	Sigma (Deisenhofen, Germany)	#E-1510
Ficoll 400	Sigma (Deisenhofen, Germany)	#F-4375
Fast Green	BDH Chemicals Ltd. (Poole, UK)	#42053
$FeCl_2$	Sigma (Deisenhofen, Germany)	#F-2130
Formic acid	Sigma (Deisenhofen, Germany)	#251364
Formaldehyde	Sigma (Deisenhofen, Germany)	#F-1268
Formamide	Merck KG (Darmstadt, Germany)	#12027
Glacial acetic acid	Sigma (Deisenhofen, Germany)	#338826
Glucose	Merck KG (Darmstadt, Germany)	#K2164137 309
Glycine	ICN Biomedicals (Eschwege, Germany)	#808822
Glycerol	ICN Biomedicals (Eschwege, Germany)	#800688
Guanidine hydrochloride	Merck KG (Darmstadt, Germany)	#12031
HCl	Merck KG (Darmstadt, Germany)	#1.00317.1000
Hepes	Sigma (Deisenhofen, Germany)	#7365-45-91
Isopropyl alcohol	Sigma (Deisenhofen, Germany)	#I-9516
KCl	Fluka AG (Buchs, Switzerland)	#60132
KH_2PO_4	Merck KG (Darmstadt, Germany)	#104873
$K_2HPO_4 \cdot 3H_2O$	Merck KG (Darmstadt, Germany)	#105099
$MgCl_2 \cdot 6H_2O$	Sigma (Deisenhofen, Germany)	#M-0250
$MgSO_4 \cdot 7H_2O$	Merck KG (Darmstadt, Germany)	#105886
Methanol	Merck KG (Darmstadt, Germany)	#1.06009.1011
β-Mercaptoethanol	Sigma (Deisenhofen, Germany)	#M-7154
Milk powder	Bio-Rad Laboratories (Munich, Germany)	#170-6404
MOPS	Sigma (Deisenhofen, Germany)	#M-8893
Na-acetat	Sigma (Deisenhofen, Germany)	#S-8625
$Na_2BO_2 \cdot H_2O_2 \cdot 3\ H_2O$	Merck KG (Darmstadt, Germany)	#1.06560.1000
NaCl	Merck KG (Darmstadt, Germany)	#1.06404.1000
Na_2EDTA	Merck KG (Darmstadt, Germany)	#1.06392.1000
Na_2CO_3	Merck KG (Darmstadt, Germany)	# 106392
$NaHCO_3$	Merck KG (Darmstadt, Germany)	#106329
$NaNO_2$	Merck KG (Darmstadt, Germany)	#106549
NaOH	Chemapol (Chech Republic)	#500360388
Na-thiosylfate	Sigma (Deisenhofen, Germany)	#S-1648
N-(1-Naphthyl)-ethylenediamine dihydrochloride	Sigma (Deisenhofen, Germany)	#N-9125
Na-vanadat	Sigma (Deisenhofen, Germany)	#S-6508
Nycodenz	Axis-Shiels PoC AS (Oslo, Norvay)	#1002424
Phenobarbital Nembutal™	Sigma (Deisenhofen, Germany)	#P-3636
Phenol Red	Sigma (Deisenhofen, Germany)	#P-0290
Peroxidase-conjugated Streptavidin	Dianova (Hamburg, Germany)	#016-030-084
PMSF	Sigma (Deisenhofen, Germany)	#P-7626
Phosphoric acid (conc.)	Merck KG (Darmstadt, Germany)	#1.00573.1000
Sodium dodecil sulphate	Bio-Rad Laboratories (Munich, Germany)	#161-0302
Sulfanilamide $C_6H_8N_2O_2S$	Sigma (Deisenhofen, Germany)	#S-9251
Sucrose	Serva (Heidelberg, Germany)	#35579
Sulfo-NHS	Sigma (Deisenhofen, Germany)	#56485
TCA $C_2HCl_3O_2$	Sigma (Deisenhofen, Germany)	#522082
TEMED	Bio-Rad Laboratories (Munich, Germany)	#161-0800
Tris-HCl	Merck KG (Darmstadt, Germany)	#108219
Triton X-100	Sigma (Deisenhofen, Germany)	#9002-93-1
Trypan Blue Solution 0.4%	Sigma (Deisenhofen, Germany)	#T-8154
Tween 20	Serva (Heidelberg, Germany)	#37470
Urea	ICN Biomedicals (Eschwege, Germany)	#821527
Xylene cyanol	Sigma (Deisenhofen, Germany)	#X-4126

2.1.2.1. Commercial reagents and kits

Table 2.2. Commercial reagents and kits

Reagent/Kit	*Company*	*Catalog No.*
Bichinonic acid protein assay kit	Sigma (Deisenhofen, Germany)	CA-1, B9643
Bio-Rad Protein Assay	Bio-Rad Laboratories (Munich, Germany)	#500-0006
Bovine serum albumin	Serva (Heidelberg, Germany)	#11925, #11930
Cytotoxicity Detection Kit	Merck KG (Darmstadt, Germany)	#1 644 793
High Pure PCR Product Purification Kit	Roche Diagnostic GmbH (Mannheim, Germany)	#1 732 668
LumiGLO® Reagent and Peroxide	Cell Signaling Technology, Inc. (Beverly, MA, USA)	#7003
Oligo $(dT)_{15}$ Primer	Promega (Mahnheim, Germany)	#C1101
Fluoresbrite™ calibration grade 1.0 micron YG Microspheres (2.66% Solids latex)	Polysciences Inc. (Warrington, PA, USA)	#18860
QIAshredder spin columns	Quiagen (Hildesheim, Germany)	#79656
Recombinant RNasin Ribonuclease inhibitor	Promega (Mahnheim, Germany)	#N2511
RNase-Free-DNAse Set	Quiagen (Hildesheim, Germany)	#79254
RNeasy kit	Quiagen (Hildesheim, Germany)	#74106
SuperScript RNase H^- Reverse Transcriptase, 1st Strand Buffer	Invitrogen Life technologies (Karlsruhe, Germany)	#18053-017
	Gibco (Karlsruhe, Germany)	# 18064-022
Taq DNA polymerase, PCR Buffer	Promega (Mahnheim, Germany)	#M1865
Taq DNA polymerase, PCR Buffer	Invitrogen Life technologies (Karlsruhe, Germany)	#10342-020
TRIzol LS Reagent (NEW)	Invitrogen Life technologies (Karlsruhe, Germany)	#10296-028

2.1.2.2. Antibiotics

Penicillin G. K-salt	Serva (Heidelberg, Germany)	#31749
Streptomycin sulfate	Serva (Heidelberg, Germany)	#35500

2.1.2.3. Enzymes

Table 2.3. Enzymes

Enzyme	*Company*	*Catalog No.*
Catalase	Sigma (Deisenhofen, Germany)	#C-9322
Collagenase (CLS)	Seromed-Biochem KG (Berlin, Germany)	#C-1-22
DNAse I from Bovine pancrease	Boehringer Mannheim (Mannheim, Germany)	#1284932
Pronase from Clostridium hislyticum	Roche Diagnostic GmbH (Mannheim, Germany)	#1 213 865

2.1.2.4. Cell culture media

Table 2.4. Cell culture media

Media	*Company*	*Catalog No.*
RPMI medium	Biochrom AG (Berlin, Germany)	#T 127-50
Newborn calf serum	Biochrom AG (Berlin, Germany)	
Fetal calf serum	BoiWest (Nuaillé, France)	
Hanks	Biochrom AG (Berlin, Germany)	#L201-05
DMEM	Biochrom AG (Berlin, Germany)	#F 0405

2.1.2.5. Stimuli

Table 2.5. Substances used for cells stimulation

Stimulus	*Company*	*Catalog No.*
Dexamethasone	Calbiochem/Merck Biosciences GmbH (Bad Soden, Germany)	#265005
Indomethacine	Sigma (Deisenhofen, Germany)	#I-7378
L-NIL	Calbiochem/Merck Biosciences GmbH (Bad Soden, Germany)	#482100
L-NMMA	Calbiochem/Merck Biosciences GmbH (Bad Soden, Germany)	#475886
LPS *(Salmonella Minnesota, R595)*	Gift from Dr. Galanos (Freiburg, Germany)	---
PGE_2	Sigma (Deisenhofen, Germany)	#P0409
PGH_2	Cayman Chemical Company (Ann Arbor, Michigan, USA)	#17020
Recombinant rat IL-1β	R&D Systems (Wiesbaden, Germany)	#501-RL
Recombinant rat IL-6	R&D Systems (Wiesbaden, Germany)	#506-RL
Recombinant rat IL-10	R&D Systems (Wiesbaden, Germany)	#522-RL
Recombinant rat TNFα	R&D Systems (Wiesbaden, Germany)	#510-RT
SC-236	Searle, Monsanto Co. (St Louis, MO, USA)	
SC-560	Searle, Monsanto Co. (St Louis, MO, USA)	
SNAP	Calbiochem/Merck Biosciences GmbH (Bad Soden, Germany)	#487910
Zymosan A. (*Sacc. Cerevisiae*)	Sigma (Deisenhofen, Germany)	#Z-4250

2.1.2.6. Antibodies

2.1.2.6.1. Primary antibodies for Western blot

Table 2.6. Primary antibodies for Western blot

Antibodies	*Origin of antigen*	*Clon (Poly/ Mono)*	*Host*	*Catalog No.*	*Company*	*Used dilutions*	M_r^*
Anti-5-Lipoxygenase	Human	Poly.	Rabbit	#160402	Cayman Chemical Company (Ann Arbor, Michigan, USA)	1:1 000	78
Anti-actin	Mouse	Mon..	Mouse	#A5316	Sigma-Aldrich Chemie Gmbh (Munich, Germany)	1 :500 1 :2 500	42
Anti-COX-1	Murine	Poly.	Rabbit	#160109	Cayman Chemical Company (Ann Arbor, Michigan, USA)	1:2 000	70
Anti-COX-2	Murine	Poly.	Rabbit	#160126	Cayman Chemical Company (Ann Arbor, Michigan, USA)	1:500	72
Anti-cPLA$_2$	Murine	Poly.	Rabbit	#PL 05	Oxfrod Biomedical research (Oxford, MI, USA), Natutec GmbH (Frankfurt am Main, Germany)	1:1 000	
Anti-EP$_1$ Receptor	Human	Poly.	Rabbit	#101740	Cayman Chemical Company (Ann Arbor, Michigan, USA)	1:500 1:1 000	42
Anti-EP$_2$ Receptor	Human	Poly.	Rabbit	#101775	Cayman Chemical Company (Ann Arbor, Michigan, USA)	1:500 1:1 000	52
Anti-EP$_3$ Receptor	Human	Poly.	Rabbit	#101760	Cayman Chemical Company (Ann Arbor, Michigan, USA)	1:500 1:1 000	53
Anti-EP$_4$ Receptor (C-term)	Human	Poly.	Rabbit	#101775	Cayman Chemical Company (Ann Arbor, Michigan, USA)	1:500 1:1 000	52
Anti-iNOS/NOS Type II	Mouse	Mono.	Mouse	#610599	BD Transduction Laboratories (San Jose, CA, USA)		130
Anti-mPGES-1	Human	Poly.	Rabbit	#160140	Cayman Chemical Company (Ann Arbor, Michigan, USA)	1:500	16
Anti-mPGES-2	Human	Poly.	Rabbit	#160145	Cayman Chemical Company (Ann Arbor, Michigan, USA)	1:200	33
Anti-PGES (cytosolic)	Human	Poly.	Rabbit	#160150	Cayman Chemical Company (Ann Arbor, Michigan, USA)	1:250	23
Anti-PGES (cytosolic)	Human	Poly.	Rabbit	--	[1)]	1:250 1:1 000	23
Anti-PGDS	Rabbit	Poly.	Rat	--	[2)]	1:1 000	26[3)]
Anti-PGF$_{2\alpha}$S (lung type)	Human	Poly.	Rabbit	--	[4)]	1:2 000	37[5)]
Anti-PGF$_{2\alpha}$S (liver type)	Cow	Poly.	Rabbit	--	[4)]	1:2 000	37[6)]
Anti-Phospho-cPLA$_2$ (Ser505)	Human	Poly.	Rabbit	#2831	Cell Signaling Technology, Inc. (Beverly, MA, USA)	1:1 000	110
Anti-TxAS	Human	Poly.	Rabbit	#160715	Cayman Chemical Company (Ann Arbor, Michigan, USA)	1:1 000	60

**Molecular weight or relative mobility on SDS-PAGE (after producer's data)*

[1)] Antibodies were kindly provided by Murakami M. (Japan)
[2)] Antibodies were kindly provided by Kanaoke and Urade (Japan)
[3)] Urade et al., 1987
[4)] Antibodies were kindly provided by Watanabe K. (Japan)
[5)] Suzuki-Yamamoto *et al.*, 1999a
[6)] Suzuki-Yamamoto *et al.*, 1999b

2.1.2.6.2. Primary antibodies for ELISA

Table 2.7. Primary antibodies for ELISA

Antibodies	*Origin of antigen*	*Stock solution*
Anti-PGE_2	Mouse	1 mg/ml in H_2O

2.1.2.6.3. Secondary antibodies

Table 2.8. Secondary antibodies for Western blot and ELISA

Antibodies	*Origin of antigen*	*Host*	*Catalog No.*	*Company*	*Used dilutions*
Anti-Biotin HRP-linked	Biotin	Goat	#7075	Cell Signaling Technology, Inc. (Beverly, MA, USA)	1 :10 000
Anti-Mouse HRP-linked	Mouse	Horse	#7076	Cell Signaling Technology, Inc. (Beverly, MA, USA)	1:2 000
Anti-Rabbit HRP-linked	Rabbit	Goat	#7074	Cell Signaling Technology, Inc. (Beverly, MA, USA)	1:2 000
Anti-Mouse Biotin-SP-conjugated	Mouse	Goat	#515-065-062	Dianova (Hamburg, Germany)	1:5 000

2.1.2.7. SDS-PAGE and DNA Standards

Table 2.9. SDS-PAGE and DNA Standards

Marker	*Company*	*Catalog No.*
pqGOLD 50bp DNA-marker	Peqlab Biotechnologie (Erlangen , Germany)	#25-2001
RNA Molecular Weight Marker III	Roche Diagnostic GmbH (Mannheim, Germany)	#1062638
Biotinylated SDS-PAGE Standards, Low Range	Bio-Rad Laboratories (Munich, Germany)	#161-0306
Biotinylated SDS-PAGE Standards, HighRange	Bio-Rad Laboratories (Munich, Germany)	#161-0311
Biotinylated SDS-PAGE Standards, Broad Range	Bio-Rad Laboratories (Munich, Germany)	#161- 0319
Prestained SDS-PAGE Standards, Low Range	Bio-Rad Laboratories (Munich, Germany)	#161-0305
Prestained SDS-PAGE Standards, HighRange	Bio-Rad Laboratories (Munich, Germany)	#161-0309
Prestained SDS-PAGE Standards, Broad Range	Bio-Rad Laboratories (Munich, Germany)	#161- 0318

2.1.2.8. Blocking peptide

Table 2.10. Blocking peptide for Western blot

Peptide	*Company*	*Catalog No.*
cPGES Blocking peptide	Cayman Chemical Company (Ann Arbor, Michigan, USA)	#360150
EP_1 Blocking peptide	Cayman Chemical Company (Ann Arbor, Michigan, USA)	#301740
EP_2 Blocking peptide	Cayman Chemical Company (Ann Arbor, Michigan, USA)	#301750
EP_3 Blocking peptide	Cayman Chemical Company (Ann Arbor, Michigan, USA)	#301760
EP_4 (C-term) Blocking peptide	Cayman Chemical Company (Ann Arbor, Michigan, USA)	#301775
TxAS Blocking peptide	Cayman Chemical Company (Ann Arbor, Michigan, USA)	#360715

2.1.2.9. Oligonucleotide primers

All primers are purchased from MWG Biotech (Ebersberg, Germany).

Table 2.11. Oligonucleotide primers for PCR

Target mRNA	*Sequence*	*GenBank accession N°*	*Expect. PCR product size, bp*	*Conditions of PCR: T_{ann}/N*	*Source*
β-actin	Forward 5'- GTGGGGCGCCCCAGGCACCA -3', Reverse 5'- CTCCTTAATGTCACGCACGATTTC -3'	NM_001101	540	60°C/ 30 cycles	1)
COX-1	Forward 5'- TGCATGTGGCTGTGGATGTCATCAA -3' Reverse 5'- CACTAAGACAGACCCGTCATCTCCA -3'	NM_008969 NM_017043	450	60°C/ 35 cycles	1)
COX-2	Forward 5'-ACTCACTCAGTTTGTTGAGTCATTC-3' Reverse 5'-TTTGATTAGTACTGTAGGGTTAATG-3'	NM_011198 S67772	583	55°C/ 35 cycles	1)
cPGES	Forward 5'- CCAAATGATTCCAAGCATAAAAGAA -3' Reverse 5'- TTCTGGTAAATCTACATCCTCATCA -3'	AY281130 BC085264	240	60°C/ 30 cycles	2)
$cPLA_2$	Forward 5'-CTTACGCCACAGAAAGTTAAAAGAT-3', Revesre 5'-TCCAAACAAGTCAGGAGTCATAAA -3'	S77829.1	309	60°C/ 30 cycles	1)
eNOS I	Forward 5'-AGACGGACCCAAGTTTCCTC -3' Reverse 5'-TGATGGCTGAACGAAGATTG -3'	AF085195	497	60°C/ 30 cycles	3)
eNOS II	Forward 5'- GGAGAAGATGCCAAGGCTGCTG-3' Reverse 5'-CTTCCAGTGTCCAGACGCACCA -3'	U02534 XM_216065	224	65°C/ 30 cycles	4)
GFAP	Forward 5'- ACATCGAGATCGCCACCTAC -3' Reverse 5'- ACATCACATCCTTGTGCTCC -3'	NM_017009	219	60°C/ 30 cycles	5)
iNOS	Forward 5'- GCTTGCCCCTGGAAGTTTCTC -3' Reverse 5'- CCGACCTGATGTTGCCACTGT -3'	AY211532 D12520 D14051 D44591 NM_012611 U26686 X76881	710	60°C/ 30 cycles	1)
mPGES-1	Forward 5'- CCCAGGTAGGCCACGGTGTGT -3' Reverse 5'- CTGCTGGTCATCAAGATGTACG -3'	AB041998 AB048730 AF280967 NM_021583	292	60°C/ 30 cycles	6)
mPGES-2	Forward 5'- ATATTGTCCGTGAGGGCAAG -3' Reverse 5'- TACACTGCCAGGTCAGCAAG -3'	XM_231144	226	59°C/ 32 cycles	7)
nNOS	Forward 5'- CTTGGTAGACCTCAGCTATGA -3' Reverse 5'- TGCCATCGAGGTCTCTGTCCA -3'	NM_052799 U67309 X59949	514	63°C/ 35 cycles	4)
PGDS	Forward 5'- GTTTTGGAGGTGGAAGGACT -3' Reverse 5'- CCAATAGAAATCTGCCCAAG -3'	AF021882 D82071 NM_031644	303	57°C/ 35 cycles	1)
$PGF_{2\alpha}$	Forward 5'- CATTGCTATGGAAGAGTCAACA -3' Reverse 5'- CCTGTCCTTCTGAAAAGATGTG -3'	AA859039	314	60°C/ 35 cycles	1)
TxAS	Forward 5'- ACCCAAGCTGATAGCAGACA -3' Reverse 5'- GTGACCATGTCAAAGGCTTC -3'	D28773 D 31798 NM_012687	555	67°C/ 35 cycles	1)

1) Unpublished (designed by Schade S.)
2) Generous gifts from Lazarus M.
3) Miethke *et al.*, 2003
4) Gilchrist *et al.*, 2002
5) Belanger *et al.*, 2002
6) Lazarus *et al.*, 2002b
7) Unpublished (designed by Bezugla Y.)

2.1.3. Buffers and solutions

2.1.3.1. Solutions for Kupffer cells primary culture

All buffers and solutions, used for Kupffer cells culture, are steril filtered prior to use. In preparations involving the isoosmotic solutions, the osmolality is determined by cryoscopy with a freezing point depression osmometer. The osmolality is adjusted to 275-300 mOsm/kg water by adjustment/reducing of sodium chloride.

- HBSS, modified, Ca^{2+}/Mg^{2+}-free (pre-prfusion buffer):

5.4	mM	KCl
0.38	mM	KH_2PO_4
122	mM	NaCl
4.2	mM	$NaHCO_3$
0.34	mM	$Na_2HPO_4 \cdot 2\ H_2O$
10	mM	D-Glucose $\cdot$ 1 H_2O

pH 7.4 (saturated by 95% O_2 and 5% CO_2), steril;

- Hanks' Balanced Salt solution (HBSS), modified (perfusion buffer):

1.3	mM	$CaCl_2 \cdot 2\ H_2O$
5.4	mM	KCl
0.38	mM	KH_2PO_4
0.4	mM	$MgSO_4 \cdot 7\ H_2O$
122	mM	NaCl
4.2	mM	$NaHCO_3$
0.34	mM	$Na_2HPO_4 \cdot 2\ H_2O$
10	mM	D-Glucose $\cdot$ 1 H_2O
1	mM	Phenol Red

pH 7.4 (by NaOH), osmolality 275-295 mOsm/kg water, steril;

- HBSS, modified, NaCl-free:

1.3	mM	$CaCl_2 \cdot 2\ H_2O$
5.4	mM	KCl
0.38	mM	KH_2PO_4
0.4	mM	$MgSO_4 \cdot 7\ H_2O$
4.2	mM	$NaHCO_3$
0.34	mM	$Na_2HPO_4 \cdot 2\ H_2O$
10	mM	D-Glucose $\cdot$ 1 H_2O

pH 7.4 (by NaOH), steril;

- Hanks medium, with HEPES:

1.3	mM	$CaCl_2 \cdot 2\ H_2O$
5.4	mM	KCl
0.38	mM	KH_2PO_4
0.4	mM	$MgSO_4 \cdot 7\ H_2O$
0.5	mM	$MgCl_2 \cdot 6\ H_2O$
122	mM	NaCl
0.34	mM	$Na_2HPO_4 \cdot 2\ H_2O$
10	mM	D-Glucose $\cdot 1\ H_2O$
20	mM	HEPES

pH 7.4, steril;

- RPMI culture medium:

1.03	% (w/v)	RPMI 1640
0.0065	% (w/v)	Penicillin
0.01	% (w/v)	Streptomycin
0.2	% (w/v)	$NaHCO_3$

pH 7.3 (by HCl conc.), osmolality 275-285 mOsm/kg water, steril;

- PBS:

138	mM	NaCl
2.68	mM	KCl
7.3	mM	$Na_2HPO_4 \cdot 2\ H_2O$
1.47	mM	KH_2PO_4

pH 7.4 (by 1M HCl), steril;

2.1.3.2. Solutions for ELISA

- $NaHCO_3$/EDTA-solution:

2	% (w/v)	$NaHCO_3$
100	mM	EDTA

- ELISA Conjugation solution:

0.2	% (w/v)	BSA
7.5	µM	ECDI
0.01	mM	Sulfo-NHS

- ELISA Buffer I:

0.05	M	$NaHCO_3$
0.05	M	Na_2CO_3

pH 6.9 (by 0.05 M Na_2CO_3), prepared immediately before use;

- ELISA Buffer II:

0.1 % (w/v)	BSA
	PBS

Prepared immediately before use;

- ELISA Washing Buffer:

0.9 % (w/v)	NaCl
5 mM	Tris
0.05 % (w/v)	Tween 20

pH 7.4;

- ELISA Substrate's solution

60 mM	$Na_2HPO_4 \cdot 2\ H_2O$
39 mM	Citric acid
2 mM	ABTS
3.25 mM	$Na_2BO_2 \cdot\ H_2O_2 \cdot 3\ H_2O$

pH 4.5, fresh prepared;

2.1.3.3. Solutions for protein lysate preparations

- Cell Lysis Buffer (for total protein extraction):

10 mM	Tris-HCl
150 mM	NaCl
2 mM	EDTA
1 % (w/v)	Triton X-100
0.1 mM	Aprotinin
1 mM	Na-orthovanadat
1 mM	PMSF (stock solution is prepared using iso-propanol)
1 mM	PMSF
10 mM	Benzamidin

- Cell Lysis Buffer (for cytosolic/membrane protein extraction) (after Ways *et al.*, 1992):

20 mM	Tris-HCl
10 mM	EDTA
5 mM	EGTA
0.1 mM	Aprotinin
1 mM	Na-orthovanadat
1 mM	PMSF (stock solution is prepared using iso-propanol)
1 mM	PMSF
10 mM	Benzamidin

- Cell Lysis Buffer (with sucrose (after Stichtenoth *et al.*, 2001*)*, for cytosolic/membrane protein extraction):

0.1	M	$K_2HPO_4 \cdot 3H_2O$
0.1	M	KH_2PO_4
1	mM	EDTA
0.25	M	Sucrose
0.1	mM	Aprotinin
1	mM	Na-orthovanadat
1	mM	PMSF (stock solution is prepared using iso-propanol)
10	mM	Benzamidin

All Cell Lysate Buffers are stored for 4 weeks at 4°C; aprotinin, Na-orthovanadat and benzamidin are added prior to use.

2.1.3.4. Solutions for determination of protein concentration

- Amido Black Assay Staining Solution:

0.5	% (w/v)	Amido Black 10B
45	% (v/v)	Methanol
10	% (v/v)	Glacial acetic acid
45	% (v/v)	H_2O

- Amido Black Assay Washing Buffer:

47.5	% (v/v)	Methanol
5	% (v/v)	Glacial acetic acid
47.5	% (v/v)	H_2O

- Amido Black Assay Dissolution Buffer:

80	% (v/v)	Formic acid
10	% (v/v)	Glacial acetic acid
10	% (v/v)	TCA

2.1.3.5. Solutions for SDS-PAGE

- 5 × SDS-Samples loading buffer

0.3	M	Tris-HCl, pH 6.8
1.7	M	SDS
50	% (v/v)	Glycerol
0.1	% (w/v)	Bromophenol Blue

- Running buffer

25	mM	Tris-HCl
192	mM	Glycin
0.05	% (w/v)	SDS

pH 8.2;

- Stacking gel (3%):

3 % (w/v)	Acrylamide/Bis
125 mM	Tris-HCl, pH 6.8
0.1 % (w/v)	SDS
0.1 % (w/v)	Ammonium persulfate
0.06 % (vv)	TEMED

- Running gel (10% or 12%):

10 or 12 % (w/v)	Acrylamide/Bis (for 10% and 12% - gel respectively)
375 mM	Tris-HCl, pH 8.8
0.1 % (w/v)	SDS
0.05 % (w/v)	Ammonium persulfate
0.05 % (vv)	TEMED

- Gradient Running gel solution I (5%):

5 % (w/v)	Acrylamide/Bis
375 mM	Tris-HCl, pH 8.8
0.1 % (w/v)	SDS
0.04 % (w/v)	Ammonium persulfate
0.04 % (vv)	TEMED

- Gradient Running gel solution II (20%):

20 % (w/v)	Acrylamide/Bis
375 mM	Tris-HCl, pH 8.8
0.1 % (w/v)	SDS
25 % (w/v)	Glycerol
0.04 % (w/v)	Ammonium persulfate
0.04 % (vv)	TEMED

2.1.3.6. Solutions for Western blot

- Blotting buffer:

50 mM	Tris-HCl pH 8.2
384 mM	Glycin
0.1 % (w/v)	SDS
20 % (v/v)	Methanol

- 1 × TBS

25 mM	Tris-HCl pH 8.0
150 mM	NaCl

- TBS-T

1 × TBS	
0.05 % (v/v)	Tween-20

- Stripping buffer:

62.5 mM	Tris-HCl pH 6.8
2 %(w/v)	SDS
10 mM	β-Mercaptoethanol

2.1.3.7. Solutions for proteins staining

- Coomassie Staining solution:

50 % (v/v)	Methanol
10 % (v/v)	Acetic acid
0.1 % (w/v)	Coomassie® Brilliant Blue G250

- Coomassie Destaining solution:

30 % (v/v)	Methanol
7 % (v/v)	Acetic acid

- Fast Green Staining solution:

0.1 % (w/v)	Fast Green
1 % (v/v)	Acetic acid

2.1.3.8. Solutions for agarose gel electrophoresis

- 10 × TBE:

0.89 M	Tris-HCl pH 8.3
0.89 M	Boric acid
0.02 M	EDTA

- Agarose gel:

2 % (w/v)	Agarose
1 × TBE	
1 µg/m	Ethidium Bromide

- 2 × Urea Gel Loading buffer

2 × TBE	
13 % (w/v)	Ficoll 400
0.01 % (w/v)	Bromphenol blue
7 M	Urea

- 5 × Gel Loading buffer I (modified after Klebe *et al.*, 1999)

0.01 % (w/v)	Phenol red
60 % (w/v)	Sucrose

- 6 × Gel Loading buffer II

15 % (w/v)	Ficoll 400
0.25 % (w/v)	Bromphenol blue
0.25 % (w/v)	Xylene cyanol

2.2. Methods

2.2.1. Kupffer cells primary culture

2.2.1.1. Isolation of Kupffer cells from rat liver

Primary cultures of Kupffer cells were obtained according to Eyhorn *et al.*, 1988.
All buffers and solutions, cell's preparation and incubation, are steril filtered and pre-heated up to 37°C prior to use.

2.2.1.1a. Isolation of non-parenchymal cells

For KC isolation, the liver of male Wistar rats (500-600 g) is used. The animals are anaesthetized by Nembutal injection and the liver is aseptically removed. The portal vein is cannulated and the liver is perfused *in situ* with 450 ml HBSS Ca^{2+}/Mg^{2+}-free (pre-perfusion buffer) at 37.5 ml/min, 37°C in a non-recirculating system to remove blood.
After the initial wash out the liver is transferred into the small keg and the recirculating perfusion *ex situ* (10 min) is initiated utilizing 70 ml of 0.5% (w/v) pronase in the HBSS (perfusion buffer, isoosmotic). The perfusate is constantly maintained at pH 7.4 using $NaHCO_3$ saturated solution. After perfusate is sucked off, the next step of recirculating perfusion (15 min) is initiated utilizing 50 ml of 0,06% (w/v) collagenase and 0,02% (w/v) DNase in the HBSS (perfusion buffer). The liver is minced to small pieces and is incubated under constant agitation by magnetic stir bar at 37°C; the perfusate pH should be kept at 7.4 by $NaHCO_3$ saturated solution.
Following digestion, the liver homogenate is transferred into the small Petri plate, debris are removed and the liver is combed out. The keg is rinsed by 70 ml of 0.1% pronase and 0.01% DNase in the HBSS (perfusion buffer) and the suspension is added to the liver. The resulting suspension is incubated under constant agitation at pH 7.4 (by $NaHCO_3$ saturated solution) during 5-10 min.
Undigested tissue is removed by filtration through a nylon mesh (100µm openings) in an ice-cold beaker. Thereafter the suspension is filled up to 200 ml with HBSS Ca^{2+}/Mg^{2+}-free and distributed into 4 centrifuge tubes.
The cells are harvested by centrifugation at 30 × *g* for 2 min at 4°C. The pellet is then suspended with HBSS Ca^{2+}/Mg^{2+}-free and the cells are sedimented by centrifugation at 500 × *g* for 8 min at 4°C (pellet I). The supernatant is dissolved with HBSS Ca^{2+}/Mg^{2+}-free and centrifuged at the same conditions (pellet II). The supernatant is discarded.

2.2.1.1b. Preparations of non-parenchymal cell fractions

To remove nonviable cells and to separate the different non-parenchymal cell types, a two-phase density centrifugation is used.
When the top phase is prepared, 43 ml of HBSS NaCl-free and 17 ml the 28.7% (w/v) Nycodenz (dissolved in HBSS NaCl-free) are mixed and divided between 6 centrifuge tubes.
The bottom phase contains non-parenchymal cells suspension, prepared as following. Top layer of the cell pellet I and cell pellet II are carefully resuspended in the DMEM containing 10% FCS and mixed with 11.8 ml of 57.4% (w/v) Nycodenz solution (in HBSS NaCl-free) and 2 mg DNase up to 40 ml. The suspension is divided into 4 centrifuge tubes (set A). The rest of cell pellet I is carefully resuspended in the DMEM containing 10% FCS and mixed with 5,9 ml of 57.4% (w/v) Nycodenz solution (in HBSS NaCl-free) and 1 mg DNase up to 20 ml. The suspension is divided into other 2 centrifuge tubes (set B).

The double layer gradient formed is carefully overlaid with 10 ml of DMEM containing 10% FCS and centrifuged at 1350 × *g* for 25 min at 4°C without applying the centrifuge brake.
After centrifugation, two layers are formed; one at the top of the 28.7%-gradient mostly containing HSC (they are mainly in the set A of tubes) and a second at the interface between the two gradients mostly containing viable hepatocytes and non-parenchymal cells of higher density (in both set A and B). The remaining pellet contains the majority of the enzymatically-injured hepatocytes.
Top layer is collected as HSC and the volume is adjusted up to 35 ml by DMEM containing 10% FCS.
The interface is collected as cell's mix, resuspended in RPMI containing 1% NCS and centrifuged at 500 × *g* for 8 min at 4°C.

2.2.1.1c. Separation of Kupffer and endothelial cells by centrifugal elutriation

Kupffer cells are further separated from viable hepatocytes and other sinusoidal cells by a modification of the centrifugal elutriation method originally described by Knook *et al.*, 1977.
The elutriation system consists of a J2-21/ME Beckman centrifuge with a JE-6B rotor equipped with a standard chamber (Beckman) and linked to a high precision peristaltic Masterflex pump.
Before elutriation procedure, the pump is calibrated by H_2O. Centrifuge, pump and connective hoses is sterilized by incubation for 30 min with 6% H_2O_2 following the incubation with aqueous 10% (w/v) Catalase solution (steril filtered) for 20 min and finally washing by aq. dest.
The elutriation is performed at a rotor speed of 3250 rpm at 4°C. RPMI containing 1% NCS is used as elutriation medium. Cell suspension is loaded into the elutriation system at a peristaltic pump speed of 18.5 ml/min.
After 200 ml of medium has passed through and a cell pellet is clearly formed into the elutriation chamber, the flow rate in the pump is increased stepwise from 18.5 to 60 ml/min and the correspondent volumes of cell suspension are collected at each point accordingly to Table 2.12.:

Table 2.12. Parameters for centrifugal elutriation of cells

Pump speed, ml/min	*Volume, ml*	*Fractions//Cells*
18.5	*200*	Discard
20.5	*50*	EC / 1st fraction
24.5	*50*	EC / 2nd fraction
27.5	*50*	EC / 3rd fraction
32.0	*100*	Cells mixture // discard
37.0	*50*	KC 1nd fraction
43.0	*50*	KC 2nd fraction
49.0	*50*	KC 3rd fraction
55.0	*50*	KC 4th fraction
> 60	*50*	KC 5th fraction
	50	KC 6th fraction

2.2.1.2. Kupffer cells primary culture

For further purification the obtained cell population further, the method of selective adherence to plastic is used. The fractions 3-6 of Kupffer cells, that are found to contain the majority of KC, are centrifuged at 300 × *g* for 10 min at 4°C. The resulting pellets are collected together, washed in RPMI containing 1% NCS and centrifuged at 300 × *g* for 10 min at 4°C. The resulting pellet is suspended in 1 ml RPMI medium containing 1% NCS.
The total cell total number and viability are determined by counting in Neubauer chamber using 0.2% Trypan blue solution (sock solution is diluted 1:2 by PBS): 50 µl of suspension is diluted 1:10 in the Trypan Blue solution and calculated as following:

$$N_{viable\ cells} = A * 10^4 * 10 * 1,$$

where A – average number of cells counted in 1 mm^2 field
10^4 – volume's conversion factor for 1 mm^2
10 - dilution factor
1 – total volume of cell suspension
Kupffer cells with viability higher of than 90% are used in experiments.
Cells are then seeded on 35 mm Falcon's plates at a density of $5*10^6$ cells/plate in 2 ml of RPMI medium containing 30% NCS. The cells are maintained in primary culture at 37°C in atmosphere with 5% CO_2 for 24 h. Thereafter old medium with non-adherent cells is replaced by fresh medium and cells are incubated for 24 h at the same conditions.

2.2.1.3. Identification of Kupffer cells

Purity of KC is assessed by their typical light microscopic appearance, phagocytic activity and peroxidase staining.

2.2.1.3a. Determination of phagocytic activity

The ability of Kupffer cells to ingest fluorescent latex particles is assessed by measuring the cell-associated fluorescence.
Kupffer cells are exposured to 0.0025% fluorescein-coupled latex particles (1 μm in diameter) for 30 min in at 37°C in Hanks medium. After the incubation cells are washed with PBS and the cell-associated fluorescence is detected by microscopy, the percentage of cells phagocytosing latex is calculated.

2.2.1.3b. Peroxidase staining

Kupffer cells are exposured to 0.05% (w/v) DAB in Hanks medium containing 0.03% H_2O_2 in at a pH value of 7.2 for 20 min at 37°C.
The DAB-reacted Kupffer cells are washed 3 times in PBS to remove unreacted substrate and examined by microscope: Kupffer cells are brawn-black, endothelial cells and hepatocytes are colourless; erythrocytes are smaller, homogenous black cells. The percentage of Kupffer cells is calculated.

2.2.1.4. Stimulations of cells

All experiments are performed with rat liver macrophages kept in primary culture in RPMI medium containing 30% NCS for 48 h.
When the immediate response is envisaged, the culture medium is replaced by fresh Hanks medium containing the stimuli and cells are incubated for the indicated times (15 min to 1 h).
Otherwise (if the long time incubation is explored) the culture medium is replaced by RPMI medium containing 10% NCS. In experiments required the short pre-treatment (15 min to 1 h) of cells, the cells are incubated for the indicated times in presence of the corresponding stimuli. Thereafter cell medium is replaced by fresh RPMI medium containing 10% NCS and the stimulus (or stimuli) required for the prolonged activation of cells. After indicated times (up to 24 h) of incubation supernatants are removed, centrifuged for 10 min at 16,000 × *g* at 4ºC and, if not assayed immediately, frozen and kept at -20ºC. Cells are washed twice in sterile phosphate-buffered saline and RNA or protein lysate are prepared as described below. If immediate processing is not performed, cells are frozen in liquid nitrogen for the further preparation.
Stimuli are provided in accordance to plan of experiment as following (Table 2.13.):

Table 2.13. Dilutions of stock solutions for cell stimulation

Stimulus	*Stock solution, storing temperature*	*Dissolving*	*Dilution in the medium, per plate*	*Final concen-tration*
A23187	10 mM in DMSO, -70 °C	1:10 in DMSO, 1:10 in Hanks	1:100	1 μM
Dexamethasone	100 mM in DMSO, -20 °C	1:50 in DMSO, 1:20 in PBS	1:100	1 μM
IL-1β	10 μg/ml, 4 °C	1:100 in PBS	1:100	1 ng/ml
IL-6	0.157 mg/ml, 4 °C	1:100 in PBS	1:100	100 ng/ml
IL-10	68 μg/ml, -20 °C	1:68 in PBS	1:100	10 ng/ml
Indomethacine	10 μg/ml, -20 °C	1:100 in PBS	1:100	1 μM
L-NMMA	50 mg/ml, -20 °C	1:50 in Aq. dest.	1:100	10 μg/ml
L-NIL	100 mg/ml, -20 °C	1:38 in Aq. dest., 1:10 in Aq. dest.	1:100	10 μM
LPS	1 mM, 4 °C	1:20 in Aq. dest	1:100	500 ng/ml
PGE_2	10 mM in Ethanol, -70 °C	1:100 in Aq. dest	1:100	1 μM
SC-236	10 mM, -20 °C	1:100 in Aq. Dest	1:100	1 μM
SC-560	10 mM, -20 °C	1:100 in Aq. Dest	1:1000	0,1 μM
SNAP	Substances, per 2 mg, -20 °C	800 μl DMSO (prepared immediately before use), 1:11.3 in the medium	1:100	10 μM
TNFα	10 μg/ml, 4 °C	1:10 in PBS	1:100	10 ng/ml

2.2.2. Determination of mediator's release in the medium

To explore the release of mediators by Kupffer cells, the liver macrophages are exposed to correspondent stimuli. At indicated time points supernatant is removed, centrifuged 10 min at 16,000 × *g* at 4ºC and, if not assayed immediately, frozen and kept at -20ºC.

2.2.2.1. Enzyme linked assay (PGE_2 / TxB_2)

The amounts of PGE_2 and TxB_2 (stable end product TxA_2) release by Kupffer cells in the media are determined by specific enzyme linked assay, type blocking enzyme linked assay (ELISA) (Fig. 2.1) (Dieter *et al.*, 1999).

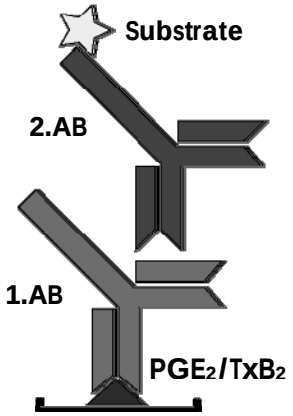

Fig. 2.1. Blocking ELISA

2.2.2.1a. Preparing of Prostanoid-BSA-complexes

PGE_2 or TxB_2 ethanol stock solution (1 mg/ml) is evaporated under N_2 and 1% (w/v) aqueous prostanoid solution is prepared. 40 µl of this is mixed with 1.6 ml of ELISA Conjugation Solution form the Prostanoid-BSA-complexes followed titration by 1 M NaOH to set pH 6.0. The dialysis tube is boiled prior to use for 10 min in $NaHCO_3$/EDTA-solution to remove the heavy metals followed washing with Aq. dist. Thereafter the dialysis of Conjugation complexes against 2 L of water for 3 h at RT under constant agitation is performed to remove uncoupled prostanoid. The final volume is adjusted to 10.4 ml by PBS (pH 7.4).

2.2.2.1b. Immobilization of prostanoid on the plate

The microtiter plates are coated using with Prostanoid-BSA-complexes (200 µl/well) diluted in the ELISA Buffer I in ratio 1:500. Plates are incubated at 4°C *ON* followed by twice washing by ELISA Washing Buffer (300 µl/well). The coat is blocked by ELISA Buffer II (300 µl/well) followed by incubation for 1 h at RT and twice washing. The plates are stored for 3 month at 4°C.

2.2.2.1c. Preparation of prostanoid calibration standards

Prostanoid (PGE_2 or TxB_2) stock solution (1 µg/ml) is diluted 1:40 and 1:6.4 for PGE_2 and TxB_2 respectively with cell culture medium and next dilutions are performed 1:2.5 as following (Table 2.14.):

Table 2.14. Dilutions of prostanoid stock solutions for ELISA

N of standard	*Final concentration, pg/100µl*	
	PGE_2	*TxB_2*
1	2 500	15625
2	1 000	6250
3	400	2500
4	160	1000
5	64	400
6	25.6	160
7	10.2	64
8	4.1	25.6
9	1.6	10.2

2.2.2.1d. Assay procedure

Background and blank values (respective cell culture medium), all standards and supernatant samples are pipetted to 96-well microtiter plates in duplicate (100 µl/well). The stock solution of Anti-PGE_2 or Anti-TxB_2 antibodies (1 mg/ml) is diluted in ratio 1:100 with ELISA Buffer II and added (100 µl/well), except the wells for background value (ELISA Buffer II instead of antibodies). The plate is incubated at 4°C *ON* followed by twice washing with ELISA Washing Buffer (300 µl/well).
Thereafter the stock solution of secondary biotin-SP-conjugated anti-mouse IgG is diluted in ratio 1:5 000 with ELISA Buffer II and assayed (200 µl/well). The plate is incubated for 1 h at RT and twice washing as described above.
ELISA substrate's solution is assayed (200 µl/well) and the plate is incubated at RT under constant shaking until the green colour appears (for 20 min – 2.5 h, in dependence of cell culture medium). The absorbance is measured by Dynatech Plate Reader at the wavelength of 405 nm. Amounts of prostanoid in the supernatant are calculated using the PGE_2/TxB_2 calibration curve prepared for each experiments.

2.2.2.3. Determination of NO

NO content in the cell supernatant is assayed using the Griess reaction (Green *et al.*, 1982), which quantifies the nitrite content; NO is an unstable molecule and degrades to nitrite and nitrate.
The calibration standards are prepared by successive dilution of $NaNO_2$ stock solution (10 mM) with Aq. dest. as following (Table 2.15.):

Table 2.15. Dilutions of nitric oxide solution for assay

N of $NaNO_2$ standard solution	*Dilution ratio*	*Final concentration*
1	1:10	1 mM
2	1:2	500 µM
3	1:10	100 µM
4	1:10	50 µM
5	1:10	10 µM
6	1:10	5 µM
7	1:10	1 µM
8	Aq. dest.	Blank

Blank value, all standards and supernatant samples are pipetted to 96-well microtiter plates in duplicate (150 µl/well). Thereafter the components of Griess reaction are successive added into each well as following: 20 µl/well of 10 mM sulphanilamide solution; 10 µl/well of 1 mM naphthalenediamine and 10 µl/well of 2 M HCl. After 10 minutes at room temperature, the optical density of the samples is measured using Dynatech Plate Reader at 550 nm. The concentration of nitrite in the samples is determined from standard curves prepared for each experiment

2.2.3. Biochemical methods

2.2.3.1. Cells viability assay

Kupffer cells are exposed to correspondent stimuli and at indicated time points supernatant is removed, centrifuged 10 min at 10,000 × *g* and 2 × 50 µl of each samples are used for assay.
Cells are washed twice with PBS and the cells lysate is prepared using Cell Lysis Buffer (with Triton), as described in Chapter 2.2.3.3. "Preparation of protein lysate"; 2 × 5 µl of each lysate samples are used.
Cells viability is determined using Cytotoxicity Detection Kit (Merck) on the 96-well microtiter plate in accordance to the manufacture's protocol. The absorbance of samples is finally measured by Dynatech Plate Reader at the wavelength of 490 nm. The ratio of LDH content in the cell and in the supernatant indicates about the level of cell's death in response of distinct stimulation comparing to control cells.

2.2.3.2. Prostanoid synthase activity in situ assay (modified after Stichtenoth et al., 2001)

Primary culture of Kupffer cells is incubated in RPMI medium containing 10% NCS in the presence of acetylsalicylic acid (1 mM). The medium should be prepared immediately before use by dissolving of 0.1802 g of acetylsalicylic acid (ASA) in 10 ml of RPMI medium containing 10% NCS to obtain the stock ASA solution (10 mM). Thereafter ASA stock solution is dissolved 1:10 in the medium and the obtained medium containing ASA (final concentration 1 mM) replaces the old culture medium, in which cells has been kept in primary culture for 48 h before use.
In experiments, required the activated macrophages, LPS (500 ng/ml) is additionally added.
After 24 h of incubation the supernatant is removed, cells are washed twice with PBS and covered with 1 ml of Hanks solution containing 10 µM of PGH_2 or the equal amount of acetone (which is solvent of PGH_2 stock solution).

After 5 min of incubation on ice, the reaction is terminated by 50 µl of mixture containing 400 mM $FeCl_2$ and 4 mM of citric acid. Thereafter supernatant is removed, centrifuged for 10 min at 16,000 × *g* and the amounts of released prostanoid are determined by ELISA.

2.2.3.3. Preparation of protein lysate

2.2.3.3a. Preparation of total protein lysate

At indicated time points of incubation cells are washed twice in sterile phosphate-buffered saline and frozen in liquid nitrogen.
When protein lysate is prepared, cells are overlaid with 80 µl of ice-cold Cell Lysate buffer (for Ø 35 mm plates). After 10 min of incubation on ice, cells are scraped from the culture dish using rubber policeman. Protein extract is transferred in a microcentrifuge tube and plates are washed with 20 µl of Lysate Buffer. The flowthrough is also added to extract and they are passed several times through a 26-gauge needle or sonicated by ultrasonic homogenizer (Sonopuls) 3 time per 10 s. Cellular debris is removed by centrifugation at 16,000 × *g* for 15 min at 4ºC and total cell lysate is obtained.
When both protein extract and total RNA are prepared from the same cells, total proteins are obtained using Trisol LS Reagent from the organic phase after DNA removing in accordance as described by supplier.

2.2.3.3b. Preparation of subcellular fractions (modified after Ways et al., 1992; Ambs et al., 1995 and Stichtenoth et al., 2001)

For membrane/cytosolic protein fraction preparation, the correspondent Cell Lysate Buffer (Triton free or sucrose Buffer) is applied to cells as described above. After cells homogenisation, crude cellular debris is removed by successive centrifugation at 1 000 × *g* for 10 min and at 10 000 × *g* for 15 min at 4ºC. The half of supernatant is obtained total protein extract and used as control.
The rest of supernatant is centrifuged at 350 000 × *g* for 30 min at 4º and supernatant is removed (cytosolic fraction). To obtain the microsomal fraction the pellet is twice washed in Cell Lysate Buffer (Triton free or sucrose Buffer) and then resuspended in the same buffer, using the volume equal to volume of cytosolic fraction.

2.2.3.4. Determination of protein concentration

The protein concentration is determined:

- by modified method of Bradford (Bradford, 1976) using Bio-Rad protein detection kit (if the lysate is prepared using Cell Lysate Buffers);
- by bicinchoninic acid assay after Smith *et al.*, 1985 using BCA assay kit (Sigma) (if the protein extract is prepared with Trisol LS reagent and contains the high concentration of SDS);

The determination by both Bradford and BCA methods is performed as described in the supplier's protocol. The absorbance of samples is finally measured at the wavelength of 490 and 562 nm respectively using Shimadzu Mini spectrophotometer (or Dynatech Plate reader, if the measurement is performed on the 96-well microtiter plate).

- by Amido Black staining after Dieckmann-Schuppert *et al.*, 1997 (if the concentration is measured in the samples are ready to apply for SDS-PAGE ant contain loading buffer and ME).

The determination by the Amido Black staining method is performed using 96-well microtiter plates. The calibration standards are firstly prepared by successive dilution of BSA stock solution (4 mg/ml) with Aq. dest. as following (Table 2.16.):

Table 2.16. Dilutions of BSA solution for assay

N of BSA standard solution	*Dilution ratio*	*Final concentration*
1	-	4 mg/ml
2	1:2	2 mg/ml
3	1:2	1 mg/ml
4	1:2	0.5 mg/ml
5	1:2	0.25 mg/ml
6	1:2	0.125 mg/ml

Blank value, all standards and protein samples are pipetted duplicate to 96-well microtiter plates containing Ø 5 mm discs of cellulose acetate sheets (3 µl/discs) directly on discs. The discs are dried using hair-drier and subsequently immersed in the Amido Black Staining Solution (5 µl/discs). Staining for 10 min at RT is followed by 4 washes (250 µl/well) by Amido Black Assay Washing Buffer for 5 min each. The discs are dried and the Amido Black Assay Dissolution Buffer is added (200 µl/well). After incubation for 50 min at 50°C (or 15 min at RT) under constant shaking the cellulose acetate sheets are dissolved yielding the resulting blue solution. The absorbance is measured using Dynatech Plate reader at the wavelength of 620 nm.

2.2.3.5. SDS-PAGE (modified after Laemmli, 1970)

Protein separation is performed by SDS-PAGE using small (Bio-Rad, gel 73×100 mm) and large (SE600, gel 160×180 mm) electrophoresis chamber. Protein samples are diluted with 5 × loading buffer, then ME is added to final concentration 4% (v/v) and samples are heated at 95 °C for 5 min.
Obtained samples are loaded onto the SDS-polyacrylamide gel, prepared by successive polymerization of running and stacking gel solutions in the gel electrophoresis unit. The concentration of acrylamide in the running gel and thickness of gel are used as following: 10%, 1,5mm thick gel –for $cPLA_2$ (Ambs *et al.*, 1995); 0.75 mm thick: 10% - for COX-1,-2 and TxAS; 12% - for PGE_2, $PGF_{2\alpha}$ and PGD_2 synthases).
The amounts of loaded proteins are 10 and 25 µg/lane for small and large gel respectively; for $cPLA_2$ investigation the double amounts of total protein are used. The both biotynilated and pre-stained molecular weight standards are applied to first and last wells at the amounts 0.5 – 1 µg.
Gels run in the Running Tris-Glycin Buffer at constant current for 10 and 17.5 mA/gel/0.75 mm thick for small and large gel respectively until the proteins are well concentrated into the stacking gel. Then 20 and 35 mA/gel/0.75 mm thick respectively are applied until the tracking dye reaches the bottom of the gel (approximately after 1 h and 2.5 h for small and large gel respectively).

When the molecular weight of proteins is determined, the gradient SDS-PAGE is applied. The gradient transition of acrylamid concentration in the running gel is used usually from 4% to 20% (w/v). The gradient running gel is poured with a model 385 Bio-Rad gradient former using 10 ml of each Gradient Running Gel Solutions of light and heavy density, containing 4% and 20 % of acrylamid respectively. The samples loading and the run conditions are as described above.

2.2.3.6. Western blot analysis

The separated proteins are electrophoretically transferred to nitrocellulose membrane using a semidry transblot apparatus.
For blotting are used 2 layers of five blotting papers and the membrane with size 50×80 and 100×140 mm for small and large gel respectively. Both membrane and blotting paper are soaked in the Blotting buffer prior to use. The transfer is performed at 1 mA/cm^2 for 2 h at RT or at 2 mA/cm^2 for 1 h at 4°C. Thereafter the membrane is washed once in TBS for 5 min and, if necessary, is stained with Fast Green, as indicated below.
Then membrane is blocked with 5% milk solution in TBS-T for 2 h at RT or for 1 h at 37°C (using orbital incubator). After five times washing with TBS-T for 5 min, the membrane is incubated for 2 h at RT or overnight at 4ºC with antibodies against target protein. The primary antibodies are diluted in TBS-T containing 5% (w/v) BSA with ratios, indicated in the Table "Primary antibodies".
Membrane is washed again five times with TBS-T for 5 min and incubated for 1 h at RT or for 30 min at 37°C (by means orbital incubator) with the appropriate horseradish peroxidase-conjugated secondary antibodies diluted in TBS-T containing 5% (w/v) of milk powder 1:2 000 and 1:10 000 for Anti-Mouse/Rabbit and Anti-Biotin antibodies respectively. Thereafter membrane is washed 5 times with TBS-T for 5 min.
Immunoreactive bands are detected using ECL kit as described in the supplier's instruction and exposed in the high performance chemiluminescence system "Genegnome" (Syngene); the image series capture is used and exposing time varies between 2-20 min. Prestained and biotinylated SDS-PAGE Standards serve as molecular weight standards.
If samples contain a few amounts of target protein or the antibodies are weak, the expose X-ray film for the same time periods is performed following by scanning of film in the through light scanner.
For quantitative analysis Western blot's images are exported as TIFF-files into ImageQuaNT® software Version 5.0 (Molecular Dynamics®) and volume quantitation is performed by calculating the integrated intensity of all pixels inside drawn objects identified specific bands of Western blot. The background correction is performed using the background values such as object or local average.
Membrane thereafter may be stripped and reprobed with a different antibody. After five times washing with TBS-T for 5 min, the membrane is incubated twice in the 60-100 ml of Stripping buffer at 50°C for 20 min each time with occasional agitation followed by blocking with 5% milk solution in TBS-T and re-probing with antibodies as described above.

2.2.3. Protein staining

If necessary, the completeness of protein blotting is visualized using Coomassie Brilliant Blue staining (untransferred proteins in the gel) and Fast Green Staining (transferred proteins on the membrane).

2.2.3.6a. Coomassie Blue staining

The gel is stained in Coomassie Staining solution for 15 min at RT under constant agitation. After thoroughly washing with Aq. dest, proteins in gel are monitored.
If necessary, gel is destained by washing twice in Coomassie Destaining solution.

2.2.3.6b. Fast Green staining

The membrane is overlaid by Fast Green Staining solution for 5 min at RT. The background staining is removed by thoroughly washing with Aq. dest, and transferred proteins on the membrane are examined. The membrane is scanned using PowerLook scanner.
Thereafter membrane is destained by washing twice with 0.2 M NaOH for 10 min and final washing with Aq. dest.

2.2.4. Methods of molecular biology

2.2.4.1. Preparation of total RNA

All equipment used for RNA preparation is RNase-free by treatment with 0.1% (v/v) DEPC solution for 24 h followed by autoclaving for 2 h. All aqueous solutions are prepared using RNAase-free water, which is either available commercially (contains in Quiagen's kit) or prepared after treatment by DEPC as described above.
At indicated time points of incubation cells are washed twice in sterile phosphate-buffered saline and frozen in liquid nitrogen. The preparation of total RNA is performed by means of either Quiagen RNeasy kit or Trisol LS reagent, if the proteins lysate is monitored simultaneously.
When Quiagen's product is used, the disruption and homogenization of cells is performed using QIAshredder spin columns. Thereafter the preparation of total RNA is performed as described in the RNeasy kit's protocol including DNA digestion with RNase free DNase.
The preparation of RNA using Trisol LS reagent is performed as described in the supplier's instruction followed by protein extract preparation.
Purified RNA are supplied with Ribonuclease inhibitor RNAsin with final concentration 1 U/µl and the RNA content and purity are measured spectrophotometrically at 260 and 280 nm using NanoDrop® apparatus and software or Pharmacia Ultrospec III spectrophotometer.

2.2.4.2. RNA agarose gel electrophoresis

For routine check the RNA integrity and quality is used the native agarose gel electrophoresis with samples, denatured by urea, followed by staining with ethidium bromide.
Diluted samples, containing 2 µl of isolated RNA (or RNA marker) and 1 × Urea Gel Loading Buffer, are denatured for 15 min at 75°C and immediately chilled thereafter. Samples are loaded in the agarose gel and resolved electrophoretically in 1 × TBE buffer at 6 V/cm, using horizontal agarose chamber. After the running dye is reached 2/3 of track, the gel is taken off and the ethidium bromide staining is visualized under UV using gel analysis system „GeneGenius" followed by processing with "GeneSnap" software.
The inspection of the 28S and 18S rRNA bands estimates the integrity and overall quality of RNA.

2.2.4.3. Design of oligonucleotide primers

The appropriate sequence of oligonucleotide primers for PCR analysis are either extracted from published works or constructed from the known cDNA sequences, using the "Primer3" (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and "BLAST" (http://www.ncbi.nlm.nih.gov/BLAST/) software.
Sequences of own constructed primers are selected:
- to have a range 17-28 of base length;
- to content no polybase sequences;
- to be un-comlementar to other primers used in the same PCR;
- to yield the PCR product with length less as 1 kb;
- to have a melting temperature in range 55-80 °C, close to T_m of other primer, used in the reaction.

The own constructed primers are monitored by sequencing of obtained PCR products. They are purified by High Pure PCR Product Purification Kit in accordance to the instruction manual. The sequencing is performed by Mrs. Glenis Wiebe (Laboratory 135, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany).

2.2.4.4. RT-PCR analysis

Total RNA in amount of 1 μg are reverse transcribed in the Perkin Elmer PCR system 2400 using SuperScript reverse transcriptase in 50 mM Tris-HCl (pH 8,3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0,5 mM of each dATP, dGTP, dCTP and dTTP and 25 μg/ml Oligo $(dT)_{15}$ Primer, as described in the supllier's instruction.
Thereafter the polymerase chain reaction is performed using Perkin Elmer PCR system 2400. The PCR mixture consists of 50-200 ng of cDNA (depending on contents of target RNA in the cell) and master mix, containing 1 U of Taq DNA polymerase, 0,2 mM of each dATP, dGTP, dCTP and dTTP, 1 μM of each specific primers, 1,5 mM $MgCl_2$, 50 mM KCl and 20 mM Tris-HCl (pH 8.4).
The cycling conditions for all reactions are: initial melt 95°C/135 s; annealing and extention 95°C/45s, T_{ann}/30s, 72°C/60s, repeated for N cycles; final extention 72°C/7 min following 4°C, where T_{ann} and N are indicated for each primer above.
To compare the levels of different mRNA in the cell, β-actin is chosen to standardize the different samples.
Both amplified cDNA fragments and DNA standards are resolved electrophoretically on 2% agarose gel and visualized by ethidium bromide, as described for RNA agarose gel. Gel Loading Buffer is added either to PCR master mix before reaction (5 × Gel Loading Buffer I) or to ready PCR products (6 × Gel Loading Buffer II).
For quantitative analysis gel's images are exported as TIFF-files and processed as described for Western blot's images.

2.2.5. Statistical analysis

The data resulting from each experimental group are expressed as means ± SEM. The significance in differences between the two distinct data groups is determined using unpaired Student's ***t*** test. A ***P*** value less 0.05 is considered statistically significant.

3. RESULTS

3.1. Prostanoid synthesis pathway in resident and LPS-stimulated Kupffer cells

3.1.1. Time-course study of PGE_2 and TxA_2 release by rat Kupffer cells after LPS and dexamethasone treatment

To assess the prostanoid production by KC during 24 h after stimulation with LPS (500 ng/ml), the amounts of PGE_2 and TxA_2, released in the culture medium, are measured at time points 2, 4, 8 and 24 h after LPS stimulation.

In resting cells, the release of prostanoids into the medium is very low (data not shown). LPS induces an enhanced release of PGE_2 and TxA_2 in liver macrophages (Fig. 3.1.A) of 0.9±0.3 pmol/10^6 cells/2 h and 38±3 pmol/10^6 cells/24 h for PGE_2 and 54±11 pmol/10^6 cells/2 h and 108±16 pmol/10^6 cells/24 h for TxA_2, respectively.

The effect of LPS differs in release rates during the 24-hours period of incubation (Fig. 3.1.B). The PGE_2 production has a lag phase of 2-4 h from a very low release (0.5±0.1 pmol/10^6cells/h) to subsequent linear increase up to 24 h (2.2±0.1 pmol/10^6 cells/h). The effect of LPS on TxA_2 production starts without a time-delay (27±6.7 pmol/10^6 cells/h) and decreases during 24 h of incubation (3.2±1.8 pmol/10^6 cells/h).

The activation of AA cascade can be inhibited by dexamethasone (Masferrer *et al.*, 1994). To examine the effect on LPS-activated Kupffer cells, cells are pre-treated with dexamethasone (1 μM) for 1 h (Table 3.1.): the glucocorticoid leads to inhibition of both PGE_2 and TxA_2 release. The effect of dexamethasone appears at the same time, when the action of LPS becomes significant (after 4 h).

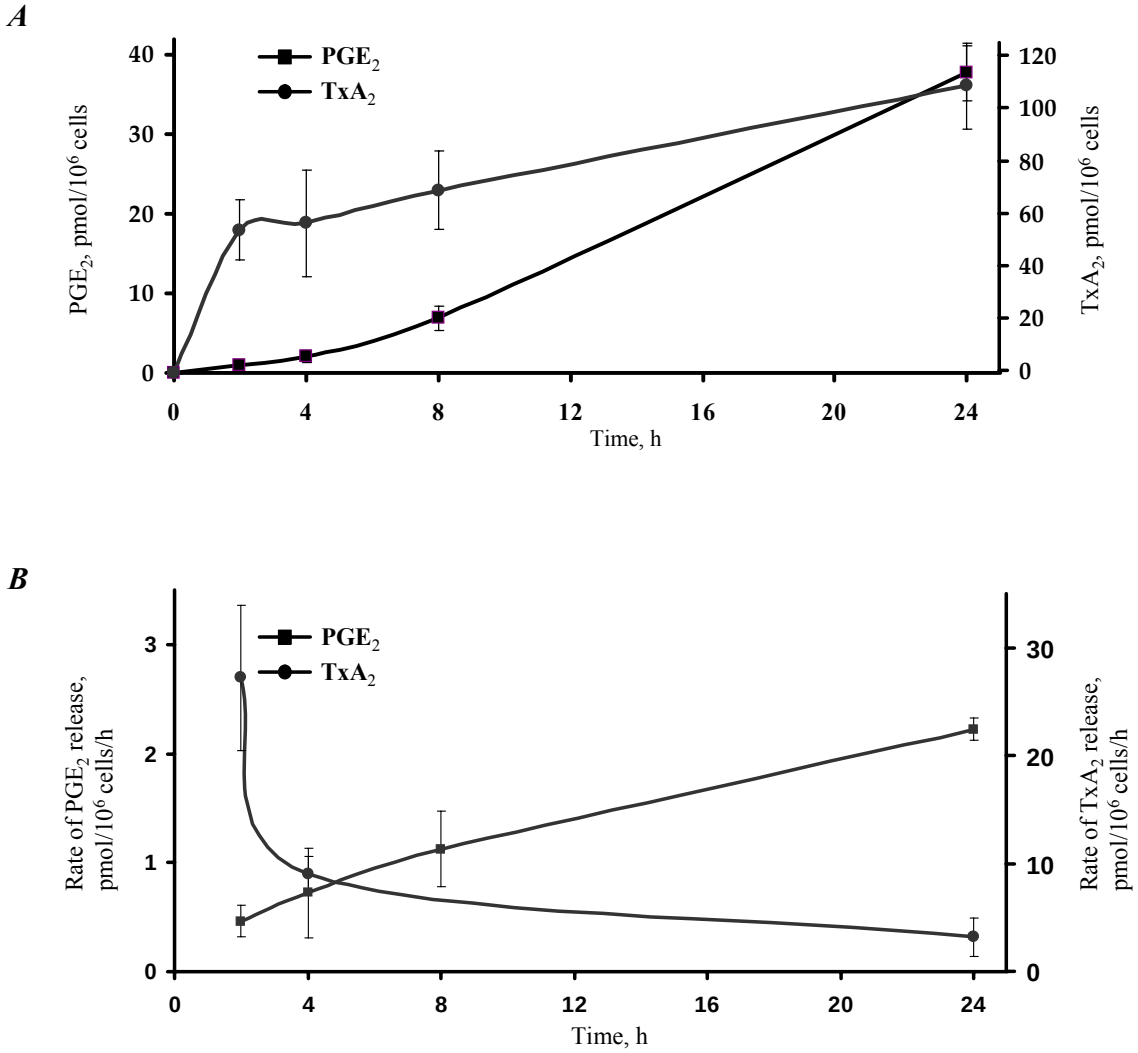

Fig. 3.1. Time course study of the PGE_2- and TxA_2-release by LPS-treated liver macrophages. Liver macrophages (primary culture) are incubated for the indicated times in presence of LPS (500 ng/ml). The amounts of PGE_2 (squares) and TxA_2 (circles) in cell media are determined by specific ELISA as described in "Material and Methods". Values are corrected for prostanoids formed by cells in the absence of LPS. Data shown are means ± S.E. of at least four independent experiments.
***(A)* Release of PGE_2 and TxA_2 during 24 h of LPS-stimulation.** The release of prostanoids by unstimulated cells is as following (in pmol/10^6 cells/2, 4,8 or 24 h): PGE_2 - 1.3±0.6 / 1.4±0.5 / 1.6±0.6 / 1.8±0.7 after 2, 4, 8 and 24 h, respectively; TxA_2 - 17.5±5.6 / 16.4±4.9 / 19.1±5.5 / 24.9±5.1 after 2, 4, 8 and 24 h, respectively.
***(B)* Rates of LPS-induced PGE_2 and TxA_2 formation.** The rate is calculated as difference in the amount of prostanoids formed between two time points divided by the time difference. Data shown are means ± S.E. of at least four independent experiments.

	% of inhibition	
	PGE_2	TxA_2
4h	87 ± 4	62 ± 10
8h	93 ± 3	69 ± 10
24h	99 ± 1	86 ± 4

Table 3.1. Effect of dexamethasone on LPS-treated liver macrophages. Liver macrophages (primary culture) are pretreated for 1 h with dexamethasone (+Dex; 1 μM) or solvent (-Dex). Then fresh medium with LPS (+LPS; 500 ng/ml) or without (-LPS) is added and macrophages are incubated for the indicated times. The amounts of PGE_2 and TxA_2 in cell media are determined by specific ELISA as described in "Material and Methods". The release of prostanoids by resting cells is (in pmol/10^6 cells/4, 8 and 24 h): PGE_2 - 1.6±0.6 / 1.6±0.6 / 2.0±0.9 after 4, 8 and 24 h and TxA_2 - 14±7 / 20±8 / 27±2 after 4, 8 and 24 h , respectively. The release of prostanoids by LPS-activated cells are (the control values are subtracted, in pmol/10^6 cells/4, 8 and 24 h): PGE_2 - 2.4±0.8 / 7.0±1.5 / 36.3±8.5 after 4, 8 and 24 h and TxA_2 - 27±17 / 57±26 / 106±28 after 4, 8 and 24 h, respectively. The values are corrected for the PGE_2 and TxA_2 formed in the absence of stimuli. Data shown are means ± S.E. of at least three independent experiments.

3.1.2. Effect of LPS and dexamethasone on the enzymes of arachidonic acid cascade

To examine the role of LPS and dexamethasone on the enzymes of the AA cascade, correspondent mRNA (Fig. 3.2.) and proteins (Fig. 3.3.) are analyzed in resident, LPS- and/or dexamethasone treated cells.

Resting Kupffer cells express certain amounts of mRNA encoding $cPLA_2$, COX-1, mPGES-2, cPGES and TxAS (Fig. 3.2., B, -LPS/-Dex/24 h), the expression of mRNA encoding COX-2 and mPGES-1 is very low (Fig. 3.2., A, -LPS/-Dex/0,4,8,24 h).

LPS strongly upregulates mRNA encoding COX-2 and mPGES-1 (Fig. 3.2., A, +LPS/-Dex/4, 8, 24 h). Densitometric data for the mRNA analysis are shown in Fig. 3.5.A and Fig. 3.7.A, respectively. LPS upregulates the expression of mRNA encoding $cPLA_2$ and mPGES-2 but does not alter mRNA encoding COX-1, cPGES, TxAS (Fig. 3.2., B, +LPS/-Dex/24 h).

Dexamethasone downregulates the LPS-enhanced expression of mRNA encoding COX-2 and mPGES-1 (Fig. 3.2., A, +LPS/+Dex/4, 8, 24 h). In resting and LPS-activated cells dexamethasone downregulates weakly the expression of mRNA encoding $cPLA_2$ and mPGES-2 but does not affect the expression of mRNA encoding COX-1, cPGES, TxAS (Fig. 3.2., B, -LPS/+Dex/24 h and +LPS/+Dex/24 h).

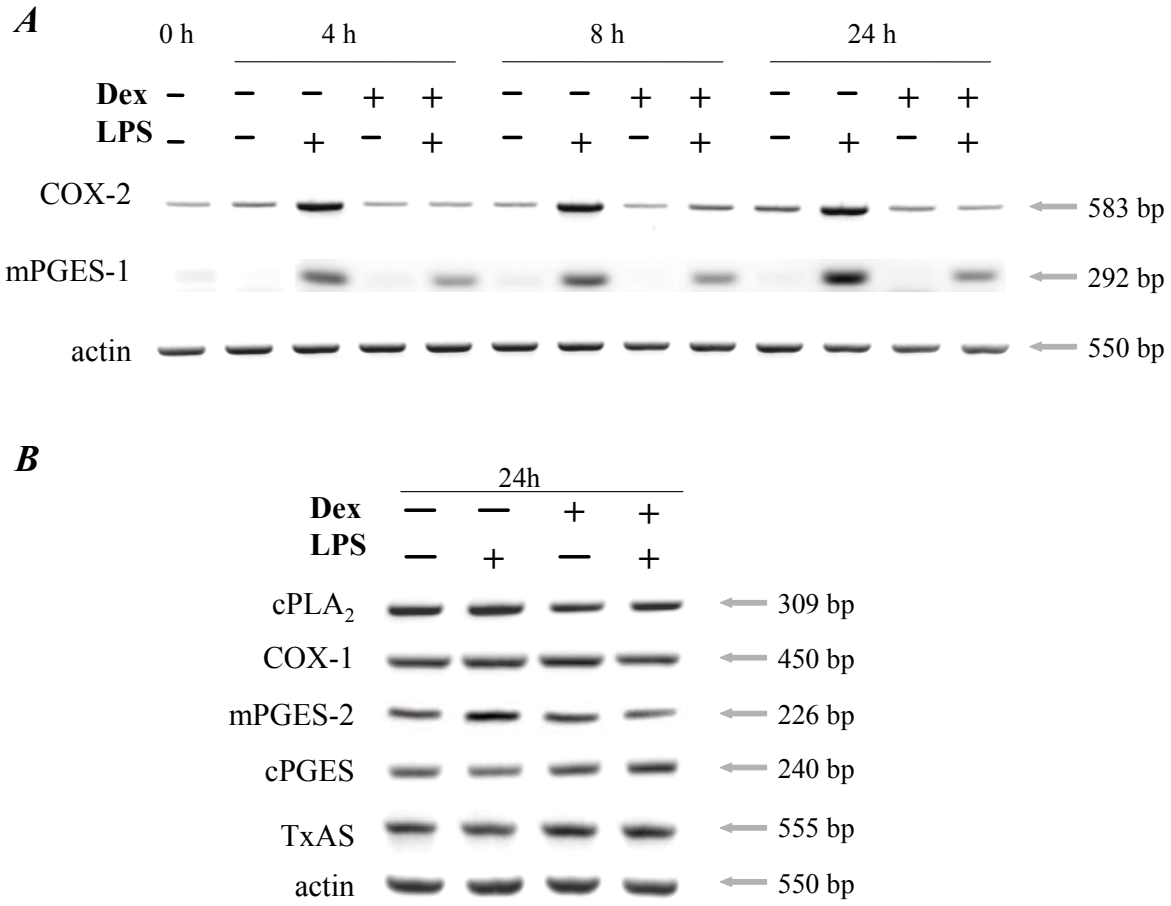

Fig. 3.2. Regulation of mRNA's transcript encoding COX-2 and mPGES-1 (A), $cPLA_2$, COX-1, mPGES-2, cPGES and TxAS (B) by LPS and dexamethasone in liver macrophages. Liver macrophages (primary culture) are pretreated for 1 h with dexamethasone (+Dex; 1 μM) or solvent (-Dex). Then fresh medium with LPS (+LPS; 500 ng/ml) or without (-LPS) is added and macrophages are incubated for the indicated times. The expression of indicated enzymes is analyzed as described in "Material and Methods". A representative set of three independent experiments is shown. The data of densitometric analysis for mRNA encoding COX-2 and mPGES-1 is shown in Fig. 3.5.A and Fig. 3.8.A, respectively; the expression of other enzymes in LPS-activated cells is as following (24 h, -Dex/-LPS corresponds 100; data are means ± S.E.): cPLA, 135±9; COX-1 104±9; mPGES-2 150±25; cPGES 98±12; TxAS 95±1.

Similar results are obtained by Western blot analysis; COX-1, mPGES-2, cPGES and TxAS proteins are expressed by resting Kupffer cells (Fig. 3.3., B, -LPS/-Dex/24 h). In contrast, the synthesis of $cPLA_2$, COX-2 and mPGES-1 proteins is very low in the absence of stimuli (Fig. 3.3., A, -LPS/-Dex/0, 4, 8, 24 h).

The expression of $cPLA_2$, COX-2 and mPGES-1 protein is enhanced when LPS is added (Fig. 3.3., A, +LPS/-Dex/4,8,24 h). In contrast, COX-1, mPGES-2, cPGES and TxAS protein expression is not changed by LPS (Fig. 3.3., B, +LPS/-Dex/24 h).

Dexamethasone suppresses the LPS-enhanced expression of COX-2, mPGES-1 and $cPLA_2$ proteins (Fig. 3.3., A, +LPS/+Dex/4, 8, 24 h). Dexamethasone does not affect the expression of COX-1, cPGES, mPGES-2, TxAS protein in resting and LPS-activated cells (Fig. 3.2., B, -LPS/+Dex/24 h and +LPS/+Dex/24 h).

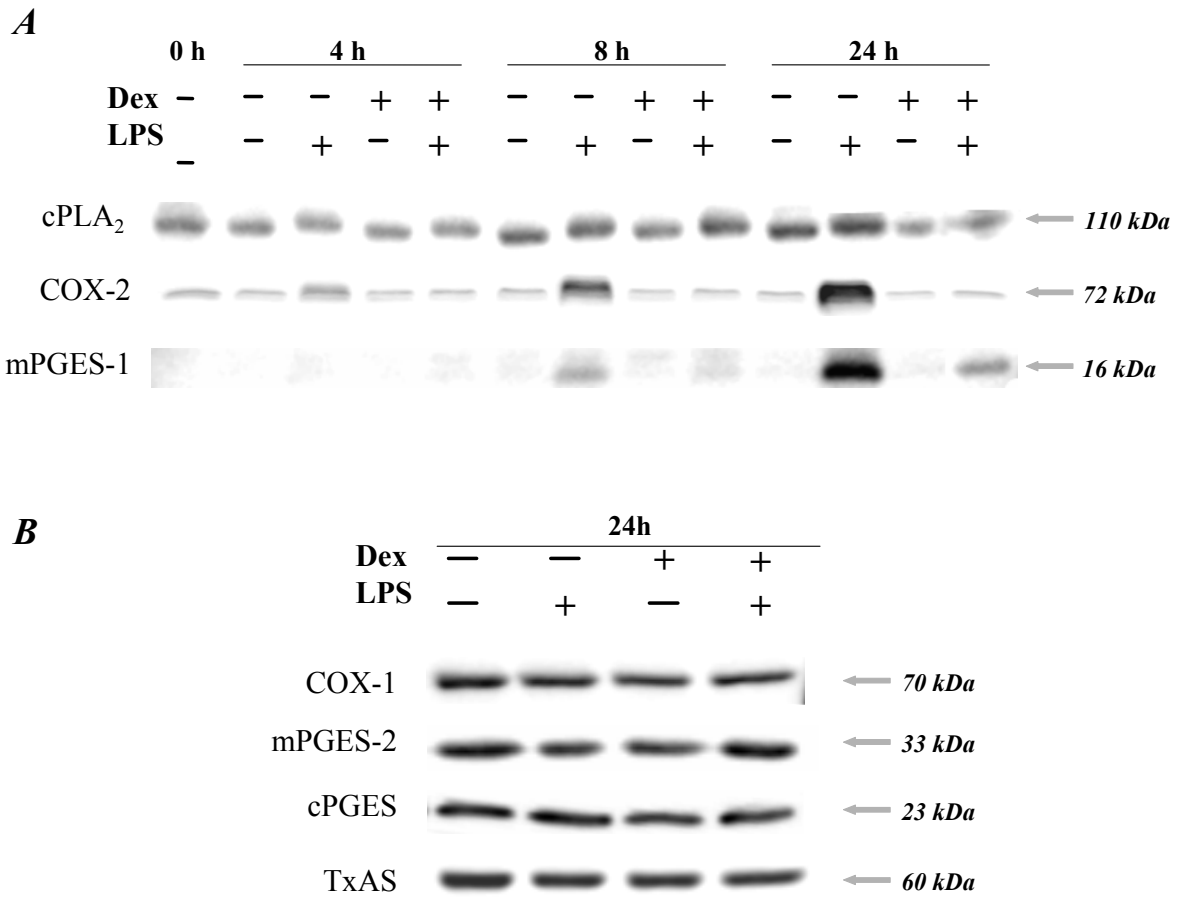

Fig. 3.3. LPS and dexamethasone regulate the synthesis of $cPLA_2$, COX-2 and mPGES-1 (*A*), but do not regulate the synthesis of COX-1, mPGES-2, cPGES, TxAS (*B*) in liver macrophages. Liver macrophages (primary culture) are pretreated for 1 h with dexamethasone (+Dex; 1 μM) or solvent (-Dex). Then fresh medium with LPS (+LPS; 500 ng/ml) or without (-LPS) is added and macrophages are incubated for the indicated times. The synthesis of indicated enzymes is analyzed as described in "Material and Methods". A representative set of three independent experiments is shown. The data of densitometric analysis for COX-2 and mPGES-1 are shown in the Fig. 3.5.B and Fig. 3.8.B, respectively; the synthesis of other enzymes in LPS-activated cells is as following (24 h, -Dex/-LPS corresponds 100; data are means ± S.E.): $cPLA_2$ 109±15 / 193±15 / 220±39 for 4/8/24 h of incubation; COX-1 95±5; mPGES-2 95±5; cPGES 106±5; TxAS 90±11.

3.1.2.1.Regulation of cytosolic phospholipase A_2

Cytosolic PLA_2 Group IV is the key enzyme, which releases arachidonic acid from phospholipids. $cPLA_2$ is expressed at a low level in resident liver macrophages (Fig. 3.2.- Fig. 3.4.). Thus, the enzyme can be rapidly (during 10 min) activated by agents (Dieter *et al.*, 2002).

LPS upregulates $cPLA_2$ mRNA expression (Fig. 3.2.B), resulting in 135%±9% of basal $cPLA_2$ expression.

The level of $cPLA_2$ protein expression (Fig. 3.3.) is about 3 fold enhanced compared by control cells (304%±58% vs. control cells) after 24 h of incubation with LPS. LPS also induces a phosphorylation of $cPLA_2$ (Fig. 3.4.).

Dexamethasone suppresses the LPS-induced expression and phosphorylation of $cPLA_2$ by 49%±6% and 72%±13%, respectively (Fig. 3.4.).

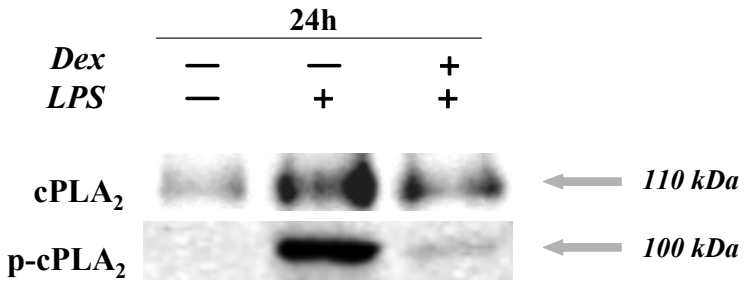

Fig. 3.4. $cPLA_2$ expression and phosphorylation in resident and LPS- and dexamethasone-treated KC. Liver macrophages (primary culture) are pretreated for 1 h with dexamethasone (+Dex; 1 µM) or solvent (-Dex). Then fresh medium with (+LPS; 500 ng/ml) or without (-LPS) is added and macrophages are incubated for the indicated times. The synthesis of indicated enzymes is analyzed as described in "Material and Methods". A representative set of three independent experiments is shown. The synthesis of $cPLA_2$ is (24 h, -Dex/-LPS corresponds 100, data are means ± S.E): 304±58 (in +LPS-activated cells), 147±28 (in +Dex/+LPS-activated cells).

3.1.2.2. Regulation of COX-1 and COX-2

Free arachidonic acid is further metabolized by cyclooxygenase isoenzymes (Smith *et al.*, 2000). Two isoforms of COX were identified in rat Kupffer cells recently (Dieter *et al.*, 2000).

The analysis of COX mRNA shows that resident liver macrophages express mRNA encoding COX-1, which is not significantly affected by LPS (Fig. 3.2.B, 104%±9% vs. control cells). Also, the expression of COX-1 protein is not influenced by LPS stimulation (Fig. 3.3.B, 95%±5% vs. control cells).

In contrast, other isoenzyme COX-2, is expressed at very little amounts in resident Kupffer cells but is strongly upregulated by LPS.

The mRNA upregulation appears already 4 h after LPS treatment and leads to a 2,5 fold higher mRNA expression during the next 20 h of incubation (Fig. 3.5.A). Addition of LPS induces also a linear increase of COX-2 protein amounts during 24 h (Fig. 3.5.B). Dexamethasone completely abrogates the LPS effect after 4, 8 and 24 h on both, mRNA (95%±3% is inhibited) and protein level (99%±1% is inhibited).

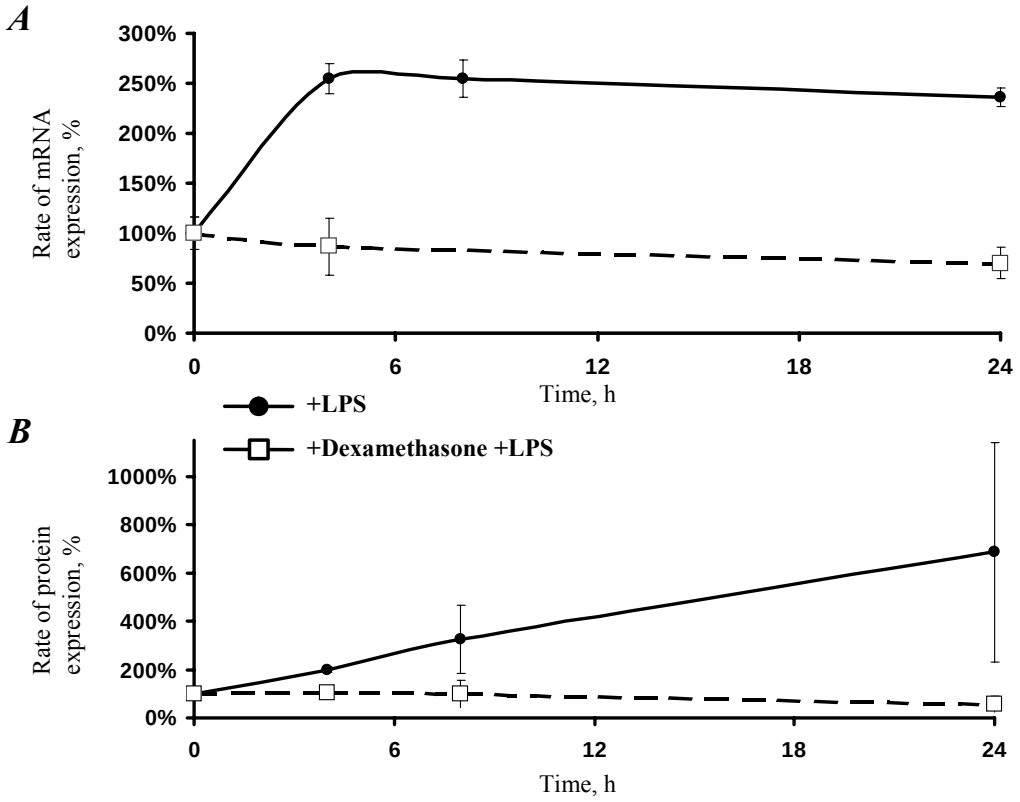

Fig. 3.5. Time-course study of COX-2: its expression *(A)* and synthesis *(B)* in LPS- and dexamethasone-treated cells. Liver macrophages are executed as described in Fig. 3.2., Fig.3.3. The quantitative analysis of mRNA ***(A)*** and proteins ***(B)*** of COX-2 is performed as described in "Materials and Methods". The values of specific bands are standardized using actin as internal standard. 24 h, -Dex/-LPS corresponds 100%. Data shown are means ± S.E. of three independent experiments.

3.1.2.3. Regulation of the final prostaglandin E_2 synthase

Recently, three final PGE_2 synthases have been described (Jakobsson *et al.*, 1999b; Tanioka *et al.*, 2000; Tanikawa *et al.*, 2002). To identify which enzymes are expressed in Kupffer cells, the analysis of mRNA encoding final PGE_2 synthases and Western blot analysis are performed. The data show that Kupffer cells express three final PGE_2-synthase isoenzymes: microsomal mPGES-1 and mPGES-2, and cytosolic PGES.

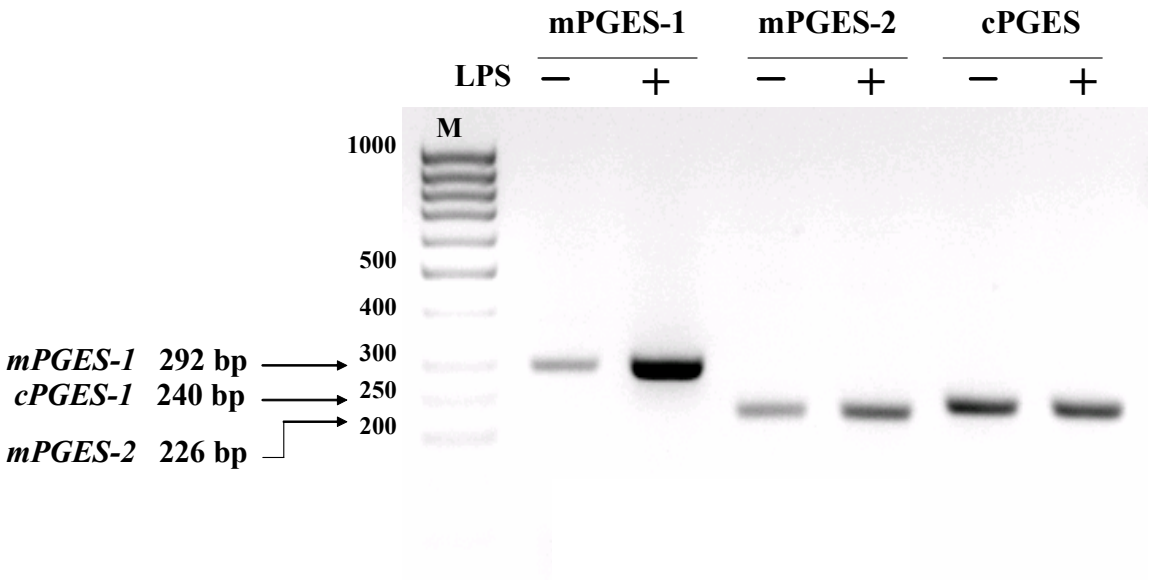

Fig. 3.6. Expression of mPGES-1, mPGES-2 and cPGES mRNA in rat liver macrophages. Macrophages (primary culture) are incubated for 24 h in presence of LPS (500 ng/ml). The expression of indicated enzymes is analyzed as described in "Material and Methods". A representative set of three independent experiments is shown. ***M*** = DNA-marker.

Resident and LPS-activated KC show the different expression of mRNA encoding three PGE_2-synthases (Fig. 3.2.A, Fig. 3.6.). The obtained PCR-products are 292, 226 and 240 bp of size for mPGES-1, mPGES-2 and cPGES respectively (Fig. 3.6.).

Resident cells express very low amounts of mRNA encoding mPGES-1 but substantial amounts of mRNA encoding mPGES-2 and cPGES. mPGES-1 is strongly upregulated by

LPS treatment (1175%±200% vs. control cells), whereas mPGES-2 shows a small increase of mRNA expression (150%±23% vs. control cells) and cPGES expression is not changed (98%±12% vs. control cells).

The kinetic of mPGES-1 mRNA expression after LPS (Fig. 3.7.A) differs from the kinetic for of COX-2 (Fig. 3.5.A): while COX-2 expression is maximal already at 4 h, the PGES-1 expression increases up to 24 h. Also, the effect of dexamethasone on mPGES-1 is different: during the first 8 h, dexamethasone inhibits the expression only by 21%±8%, after 24 h the inhibition is 83%±9%.

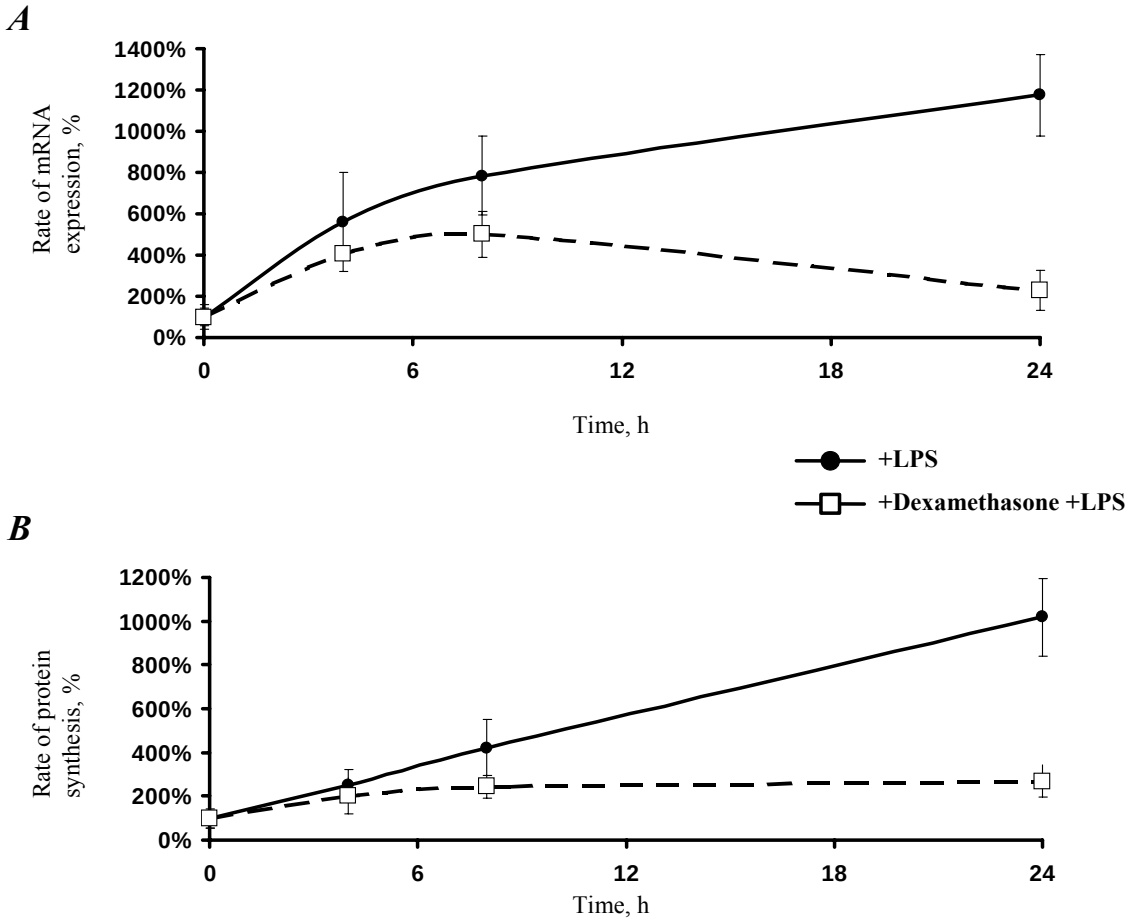

Fig. 3.7. Time-course study of mPGES-1: its expression *(A)* and synthesis *(B)* in LPS- and dexamethasone-treated cells. Liver macrophages are executed as described in Fig. 3.2., Fig.3.3. The quantitative analysis of mRNA ***(A)*** and proteins ***(B)*** of mPGES-1 is performed as described in "Materials and Methods". The values of specific bands are standardized using actin as internal standard. 24 h, -Dex/-LPS corresponds 100%. Data shown are means ± S.E. of three independent experiments.

3.1.2.4. Localization of the final PGE_2-synthases

For subcellular distribution, Western blot analysis is performed with cell lysates and with both microsomal and cytosolic fractions (Fig. 3.8.).

mPGES-1 (after LPS stimulation) is found almost totally in the microsomal fraction (98%±2% vs. total cell lysate, Fig. 3.8.A). mPGES-2 (control and after LPS stimulation) is found by 75%±2% in the microsomal fraction (Fig. 3.8.B). cPGES (control and after LPS stimulation) is almost completely found in the cytosolic fraction (Fig. 3.8.C) .

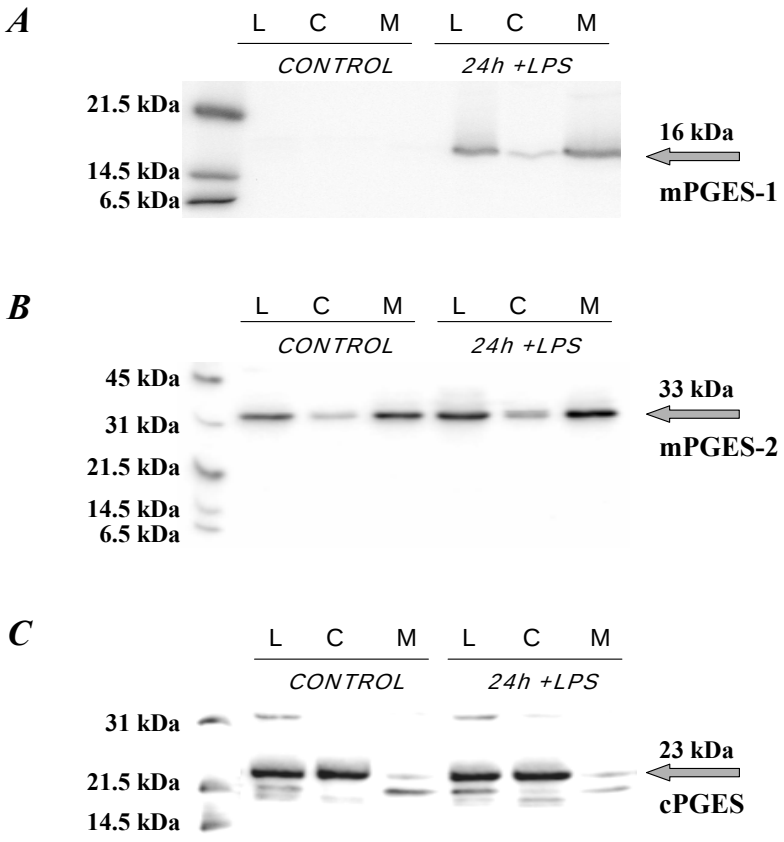

Fig. 3.8. Analysis of the subcellular distribution of mPGES–1 (*A*), mPGES-2 (*B*) and cPGES (*C*) in resident and LPS-treated KC. Cells are treated for 24 h with LPS (+LPS, 500 ng/ml) or vehicle (control). Cells lysate and subcellular fractions are analyzed as described in "Materials and Methods". A representative set of three independent experiments is shown. ***L*** = total cell lysate, ***C*** = cytosolic fraction, ***M*** = microsomal fraction.

3.1.2.5. TxA_2 synthase cellular activity (in situ)

TxA_2-synthase activity is measured in control and LPS-treated cells (Fig. 3.9.). LPS-stimulated and control cells are kept with acetylsalicylic acid in order to block the production of PGH_2 by COX isoenzymes. Thereafter, PGH_2 is added exogenously.

Fig. 3.9. shows that the release of TxA_2 in both activated and control cells after PGH_2 is about the same (96%±8% vs. control cells).

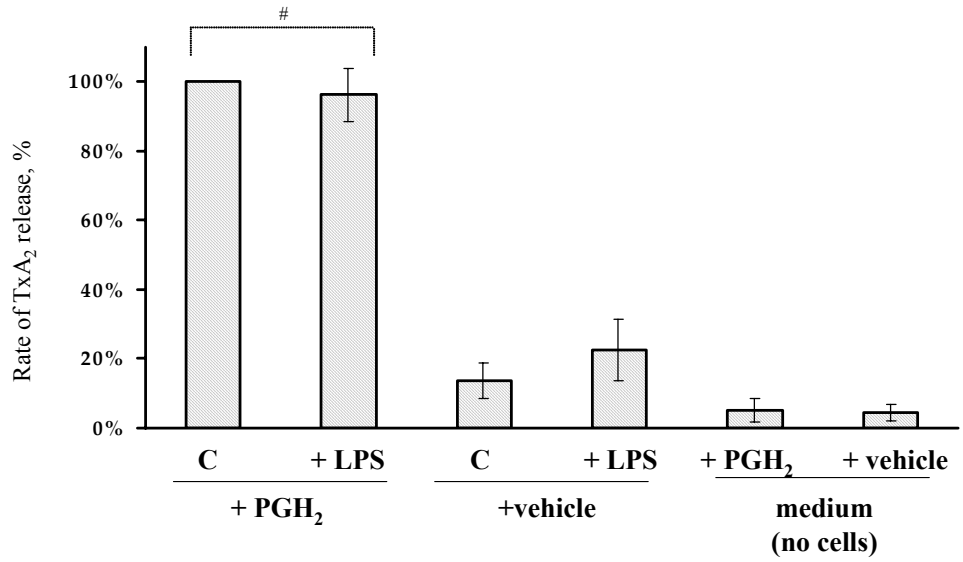

Fig. 3.9. The activity of TxA_2-synthase in resident and LPS-activated KC. Liver macrophages (primary culture) are incubated for 24 h in presence (+LPS) or absence (C) of LPS (500 ng/ml) in the medium containing acetylsalicylic acid (1 mM). TxA_2-syntase activity assay is performed and the amounts of TxA_2 are measured as described in "Material and Methods". The means ± S.E. of at least four independent experiments are shown. The release of TxA_2 by PGH_2-treatment resident cells is 186±51 pmol/10^6 cells (corresponds 100%). $^{\#}\boldsymbol{P}$>0.05 vs. control cells.

3.1.2.6. Localization of enzymes of the AA cascade

$cPLA_2$, COX-1, COX-2 and cPGES-1, mPGES-1, mPGES-2 and TxA_2-synthase show a different distribution between the cytosolic and membrane fraction in LPS-treated macrophages after 24 h of incubation (Fig. 3.10.). In detail, $cPLA_2$ and mPGES-1 are localized only in the membrane fraction; COX-1, COX-2, mPGES-2 and TxAS are found to 80% in the membrane fraction and to 20% in the cytosolic fraction. cPGES is localized mainly in cytosol (92%).

The mentioned membrane enzymes are able to shift to the subcellular fractionation in the presence of Triton-X, solubilising the association to cellular membrane (data not shown).

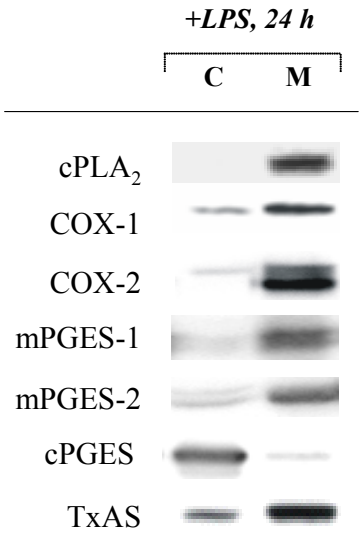

Fig. 3.10. Localization of enzymes in LPS-activated Kupffer cells. Cells (primary culture) are incubated for 24 h in presence of LPS (500 ng/ml). Subcellular fractions are executed and the synthesis of indicated enzymes is analyzed as described in "Materials and Methods". A representative set of three independent experiments is shown. The data of densitometric analysis are (cyt : mem, %): $cPLA_2$ 0:100; COX-1 20:80; COX-2 10:90; mPGES-1 0:100; mPGES-2 25:75; cPGES 92:8; TxAS 20:80. ***C*** = cytosolic fraction, ***M*** = microsomal fraction

3.1.3. Inhibition of LPS-induced PGE_2 and TxA_2 release by specific COXs inhibitors

To estimate the role of COX-1 and COX-2 in prostanoid production specific COX-1 and COX-2 inhibitors (Dieter *et al.*, 2000) SC560 and SC236 are used (Table 3.2.). The effect of inhibitors is measured after 8 h and 24 h of KC incubation. The inhibition of PGE_2 at earlier time points could not be determined because of the very low PGE_2 release.

The COX-1 inhibitor SC560 suppresses the PGE_2- and TxA_2-release after LPS at 8 h and 24 h (Table 3.2.A). The COX-2 inhibitor SC236 shows a different effect: at 8 h the PGE_2- and TxA_2-release is inhibited by 77% and 20% respectively; after 24 h, PGE_2–release is decreased to 42%, whereas TxA_2-release is increased to 34%.

The COX-non-specific inhibitor indomethacin inhibits both PGE_2 and TxA_2 release almost completely (Table 3.2.B).

A

	% of inhibition	
	PGE_2	
	8h	24h
SC-560	**91,0 ± 1,9****	**95.5 ± 1.5****
SC-236	**76.8 ± 4.1**	**42.0 ± 10.5**
	TxA_2	
	8h	24h
SC-560	**85.5 ± 6.0***	**91.6 ± 2.2***
SC-236	**20.0 ± 7.9**	**34.3 ± 3.6**

B

	% of inhibition
	indomethacin/LPS, 24 h
PGE_2	**94.1 ± 3.3**
TxA_2	**85.4 ± 4.4**

Table 3.2. Effect of COX inhibitors on the LPS-induced release of PGE_2 and TxA_2 by liver macrophages. (*A*) Effect of specific inhibitors of COX-1 (SC560) and COX-2 (SC236) on the LPS- induced release of PGE_2 and TxA_2 from liver macrophages. Liver macrophages (primary culture) are pretreated for 1 h with without/with 0,1 μM SC560 or 1 μM SC236. Then fresh medium with LPS (500 ng/ml) is added and macrophages are incubated for the indicated times. The amounts of PGE_2 and TxA_2 are determined by specific ELISA as described in "Material and Methods". Data shown are means ± S.E. of at least three independent experiments. The release of prostanoids without inhibitors in LPS-pretreated cells after 8h / 24h is 19±2/64±6 and 49±6/53±2 pmol/10^6 cells/8 or 24 h, respectively. * ***P***<0,001, ** ***P*** <0,01 (COX-1 inhibition vs. COX-2 inhibition).

(*B*) Suppression of prostanoid synthesis by indomethacin. Liver macrophages (primary culture) are pretreated for 1 h with without/with indomethacin (1 μM). Then fresh medium with LPS (500 ng/ml) is added and macrophages are incubated for 24 h, the amounts of PGE_2 and TxA_2 are determined. Data shown are means ± S.E of at least five independent experiments. The release of prostanoids without inhibitors in LPS-treated cells after 24 h is 20±1 and 37±4 pmol/10^6 cells/24 h.

3.2. Effects of cytokines on resident and LPS-activated KC

3.2.1. Effect of IL-1β on prostanoid release

Recombinant rat IL-1β induces an enhanced release of PGE_2 and TxA_2 in liver KC (Fig. 3.11.).

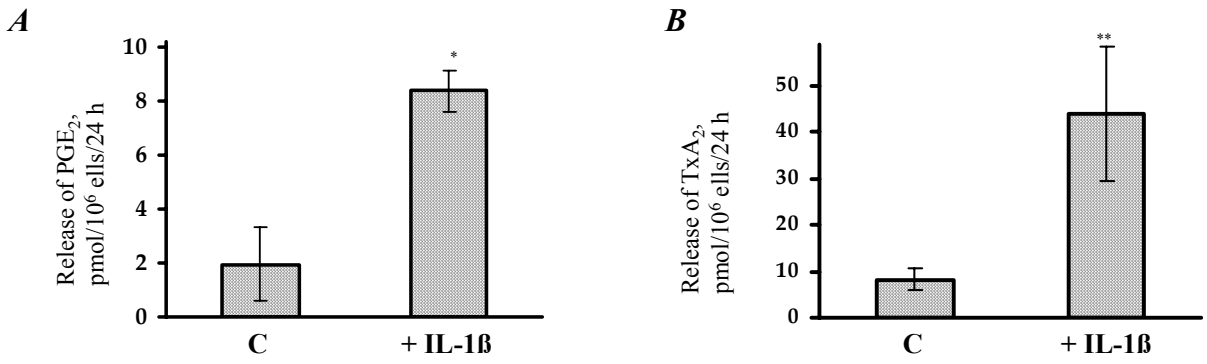

Fig. 3.11. Release of PGE_2 *(A)* and TxA_2 *(B)* after IL-1ß treatment. Liver macrophages (primary culture) are incubated for 24 h in presence (+IL-1ß) or absence (C) of IL-1ß (5 ng/ml). The amounts of PGE_2 *(A)* and TxA_2 *(B)* are determined by specific ELISA as described in "Material and Methods". Data shown are means ± S.E. of at least three independent experiments. $^{*}P<0.005$ vs control, $^{**}P<0.05$ vs. control (C).

3.2.2. Effect of TNFa on prostanoid release

Recombinant rat TNFα induces a PGE_2 and TxA_2 release in liver KC (Fig. 3.12.).

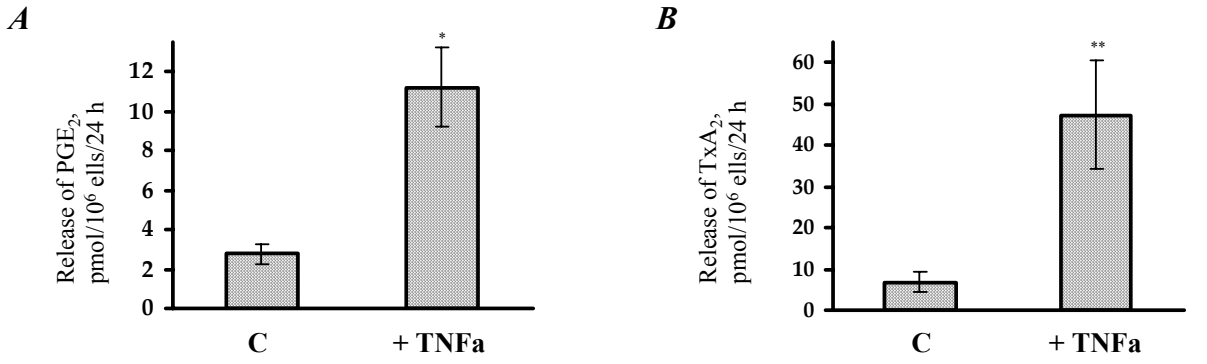

Fig. 3.12. Release of PGE_2 *(A)* and TxA_2 *(B)* after TNFa treatment. Liver macrophages (primary culture) are incubated for 24 h in presence (+TNFa) or absence (C) of TNFa (10 ng/ml). The amounts of PGE_2 *(A)* and TxA_2 *(B)* are determined by specific ELISA as described in "Material and Methods". Data shown are means ± S.E. of at least three independent experiments. $^{*}P<0.01$ vs. control, $^{**}P<0.05$ vs. control (C).

3.2.3. Synergistic effect of IL-1β and TNFa on prostanoid release by resident KC

The study of cytokine action on the macrophages cannot be profound, if it does not consider the presence of other biologically active agents. For example, during liver injury a number of inflammatory cytokines is co-produced (Gross *et al.*, 1983). The cytokine network is more important in case of IL-1β and TNFα, because a synergism between these cytokines has been reported (Dayer, 2002; Romero *et al.,* 1996).

To explore a possible synergistic effect of IL-1β and TNFα on PGE_2 and TXA_2 release by KC (Fig. 3.13.), cells are treated simultaneously by both cytokines. After 24 h of incubation the released amounts of PGE_2 are by 15% and 40% higher compared to TNFα and IL-1β alone, respectively (Fig. 3.13.A).

In case of TxA_2, a synergistic effect is observed for IL-1β (~35% higher compared to IL-1β alone), but not for TNFα (Fig. 3.13.B).

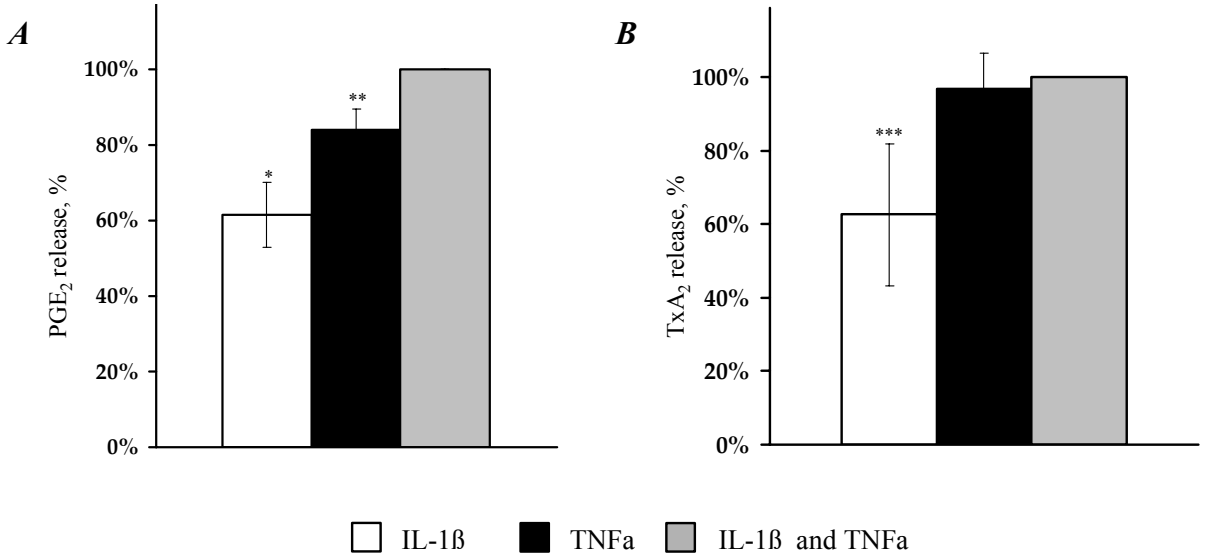

Fig. 3.13. Synergistic effect of IL-1ß and TNFa on PGE_2 *(A)* and TxA_2 *(B)* release. Liver macrophages are kept for 24 h in the medium containing rat IL-1ß (5 ng/ml) or/and rat TNFa (10 ng/ml). The amounts of PGE_2 (**A**) and TxA_2 (**B**) are determined by specific ELISA as described in "Material and Methods". The release of PGE_2 in +IL-1ß/+TNFa-activated cells is 9.9±2.8 pmol/10^6 cells/24 h (corresponds 100% of PGE_2 release), TxA_2 - 27.0±8.7 pmol/10^6 cells/24 h (corresponds 100% of TxA_2 release). Data shown are means ± S.E. of at least three independent experiments. $^{*}P<0.001$ / $^{**}P<0.01$ / $^{***}P<0.05$ vs. +IL-1ß/+TNFa-activated cells.

3.2.4. Effect of cytokines on inducible enzymes of the AA cascade in Kupffer cells

To determine whether the effect of IL-1β and TNFα occurs via changes at the transcriptional/translational level of enzymes, the analysis of mRNA and protein expression of enzymes of AA cascade is performed (Fig. 3.14.).

IL-1β and TNFα upregulate differently the mRNA expression encoding inducible enzymes involved in PGE_2 and TxA_2 production (Fig. 3.14.A). In detail, the cytokines induce a weak induction of $cPLA_2$ mRNA expression (up to ~150-170% vs. control cells). The expression of mRNA encoding COX-2 is enhanced differently: by IL-1β up to ~280%, by TNFα up to ~450% and by both cytokines up to ~560% compared to control cells.

The expression of mRNA encoding mPGES-1 is almost not affected by IL-1β (~107% vs. control cells), but it is ~2.5 times higher when cells are treated by TNFα or TNFα+IL-1β.

The expression of mPGES-2 mRNA is slightly induced by all stimuli, giving ~120% of mRNA level compared with control cells.

The synthesis of $cPLA_2$, COX-2 and mPGES-1 proteins is also upregulated by cytokines; mPGES-2, in turn, is not altered by cytokines as well as by LPS.

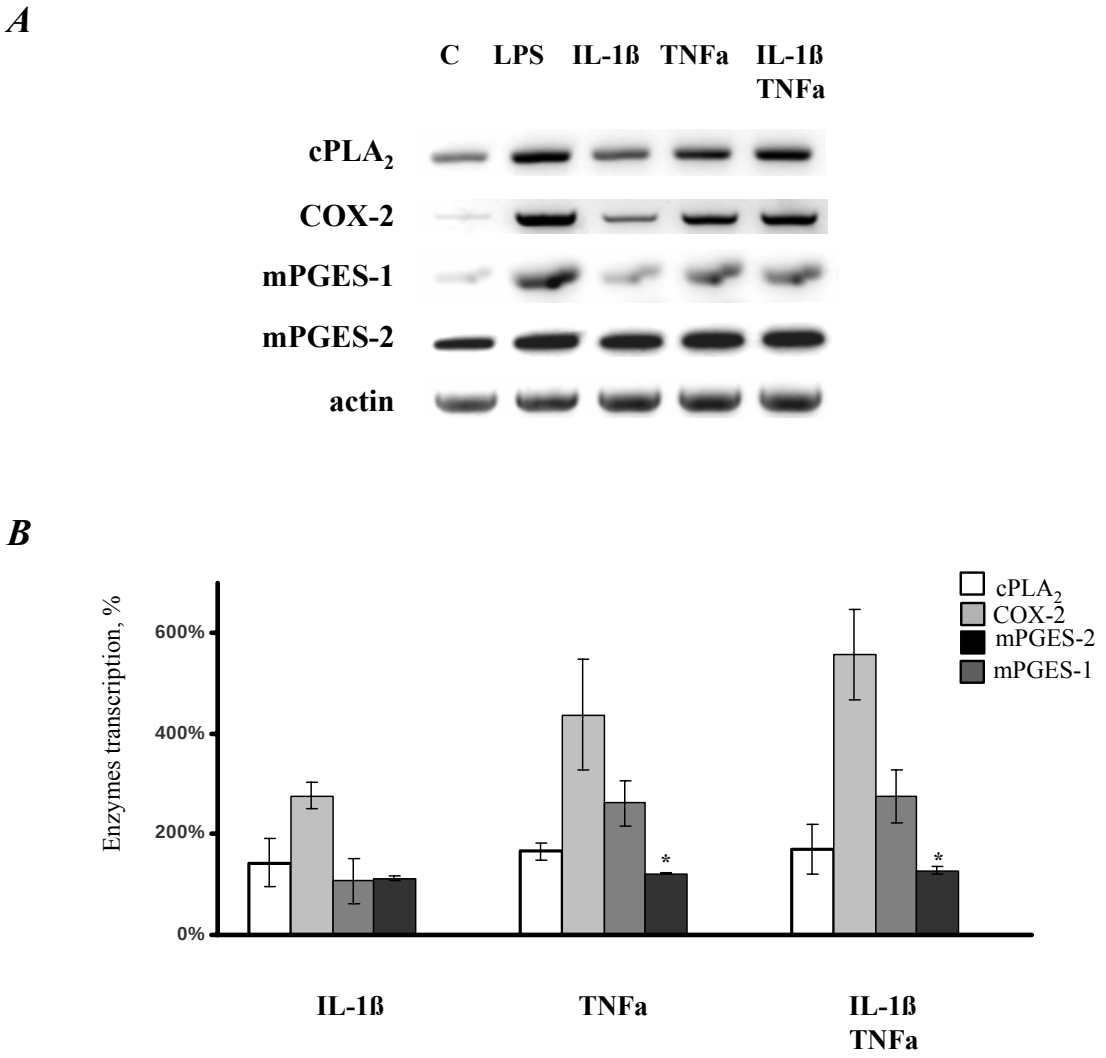

Fig. 3.14. Effect of IL-1ß and TNFa on the expression of enzymes. Liver macrophages are incubated for 24 h in the medium containing rat IL-1ß (5 ng/ml) or/and rat TNFa (10 ng/ml) as well as LPS (500 ng/ml), and in the stimuli-free medium (C). The expression of indicated enzymes is analyzed as described in "Material and Methods". A representative set of three independent experiments is shown (***A***). The rates of enzyme's induction after IL-1ß/TNFa co-incubation for mRNA encoding $cPLA_2$, COX-2, mPGES-1,-2 is shown (***B***). 100% corresponds the expression of enzymes in control cells (C); *n=2.

3.2.5 Effect of anti-fibrogenic cytokine on prostanoid release by KC

3.2.5.1 Effect of IL-6 on prostanoid production by resident KC

To examine, whether IL-6 has an influence on the PGE_2 release by liver macrophages, cells are pre-treated with recombinant rat IL-6 (to final concentration 100 ng/ml) (Fennekohl *et al.*, 2000) (Fig. 3.15.).

IL-6 has no significant effect on PGE_2 release in control cell or cells treated with LPS, IL-1β and TNFα.

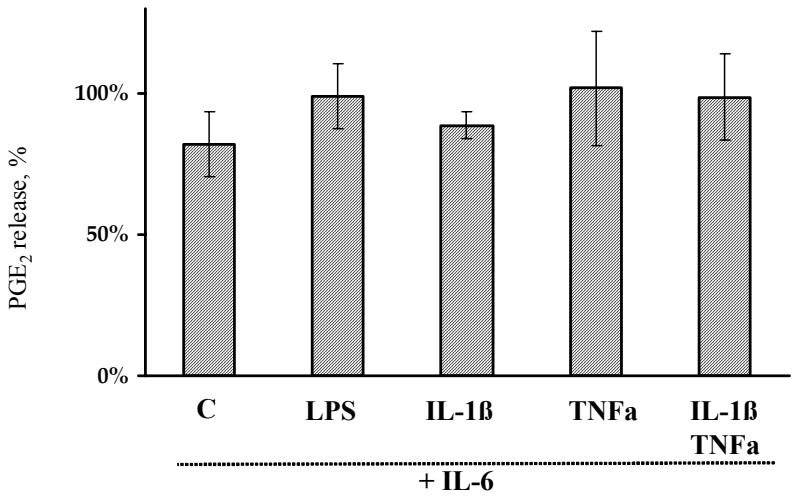

Fig. 3.15. Effect of IL-6 on PGE_2 release by LPS- and cytokines-activated Kupffer cells. Liver macrophages (primary culture) are pretreated for 1 h with IL-6 (100 ng/ml) or vehicle. Then fresh medium with LPS (500 ng/ml), rat IL-1ß (5 ng/ml), rat TNFa (10 ng/ml) and stimuli-free medium is added and macrophages are incubated for the next 24 h. The amounts of PGE_2 in cell media are determined by specific ELISA as described in "Material and Methods". Data shown are means ± S.E of three independent experiments. 100% corresponds the release of PGE_2 in absence of IL-6 (in pmol/10^6 cells/24 h): 2.5±0.5 in untreated cells (C); 8.9±1.1 in LPS-treated, 8.2±0.7 in IL-1ß-treated, 9.7±2.5 in TNFa-treated and 14.4±0.6 in combined (IL-1ß+TNFa)-treated Kupffer cells. P>0.05 vs. IL-6-untreated cells for all stimuli.

3.2.5.2 Effect of IL-10 on prostanoid production by LPS-activated KC

IL-10 does not effect LPS-induced PGE_2 and TxA_2 release after 8 h, but suppresses the release of PGE_2 and TxA_2 after 24 h by ~70% and ~50%, respectively (Fig. 3.16.).

IL-10 also decreases the expression of enzymes (Fig. 3.17.): the expression of mRNA encoding $cPLA_2$, COX-2 and mPGES-1 is downregulated to 77%±6%, 33%±8% and 60%±7%, respectively, compared to LPS-activated cells.

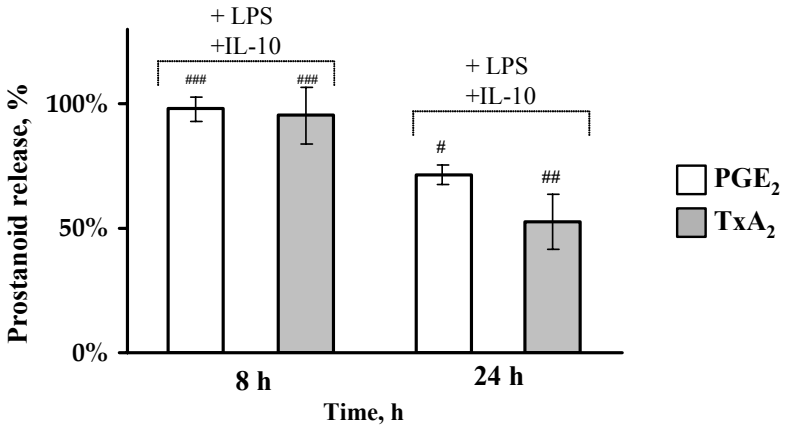

Fig. 3.16. IL-10 suppresses the release of PGE_2 and TxA_2 by LPS-activated Kupffer cells after 24 h of incubation. Liver macrophages (primary culture) are pretreated for 1 h with IL-10 (10 ng/ml) or vehicle. Then fresh medium with/without LPS (500 ng/ml) is added and macrophages are incubated for the next 24 h. The amounts of PGE_2 and TxA_2 in cell media are determined by specific ELISA as described in "Material and Methods". Data shown are means ± S.E. of at least four independent experiments. $^{\#}\boldsymbol{P}<0.001$ vs LPS, $^{\#\#}\boldsymbol{P}<0.005$ vs LPS, $^{\#\#\#}\boldsymbol{P}>0.05$, not significant. The release of prostanoids in LPS-treated cells corresponds 100% and is measured as following (in pmol/10^6 cells/8 or 24 h): PGE_2 - 18.6±6.6 and 36.7±11.2 after 8 h and 24 h, respectively; TxA_2 – 49.7±14.5 and 79.4±18.7 after 8 h and 24 h, respectively. The release of prostanoids in control and IL-10-treated cells is as following (in pmol/10^6 cells/8 or 24 h): PGE_2 - 4.4±0.8 and 6.6±3.4 after 8 h, respectively; 4.5±1.4 and 3.5±1.3 after 24 h, respectively; TxA_2 – 12.3±8.4 and 6.7±3.8 after 8 h, respectively; 16.1±7.8 and 15.8±5.1 after 24 h, respectively.

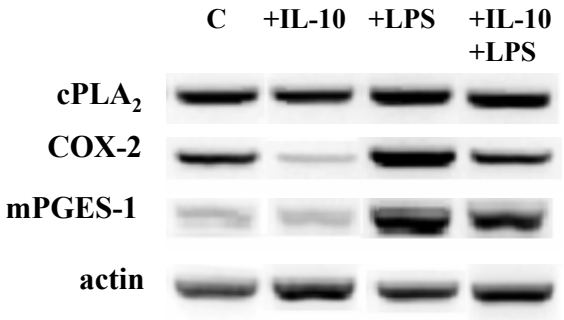

Fig. 3.17. Effect of IL-10 on expression of mRNA encoding $cPLA_2$, COX-2 and mPGES-1 in LPS-treated macrophages. Liver macrophages (primary culture) are pretreated for 1 h with IL-10 (10 ng/ml) or vehicle. Then fresh medium with/without LPS (500 ng/ml) is added and macrophages are incubated for the next 24 h. The expression of indicated enzymes is analyzed as described in "Material and Methods". A representative set of three experiments is shown. The expression of $cPLA_2$, COX-2 and mPGES-1 by IL-10/LPS-treated cells are 77±6, 33±8 and 60±7, respectively (24 h, +LPS corresponds 100). C=unstimulated cells

3.3. Effect of PGE_2 on the release of other prostanoid and cytokines

Exogenously added PGE_2 reduces TxA_2 release after LPS (Fig. 3.18.A), but potentiates the TxA_2 release by IL-1β and TNFα (Fig. 3.18.B).

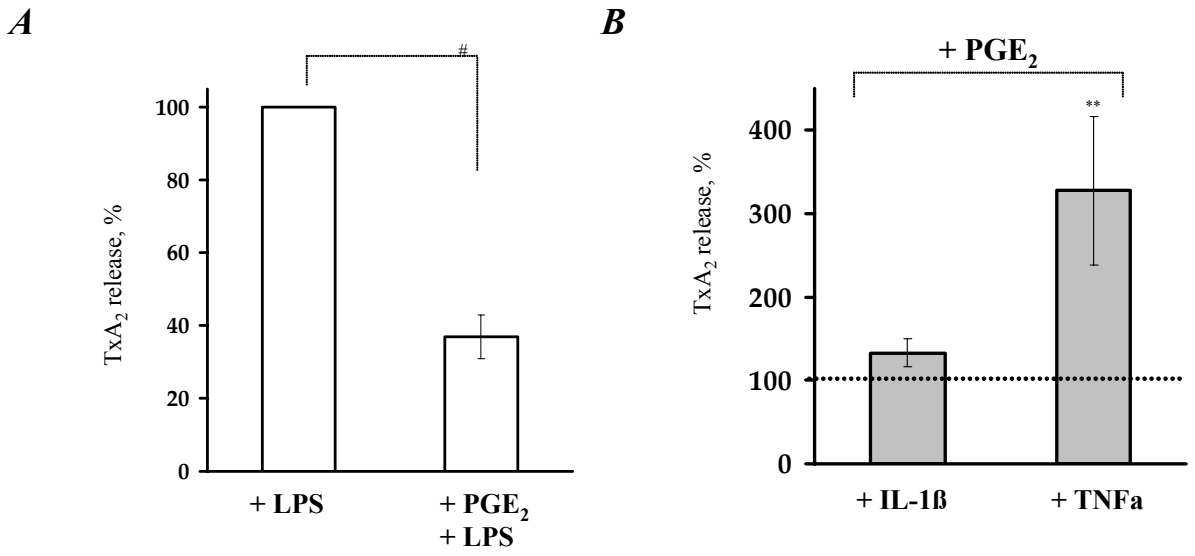

Fig. 3.18. Effect of PGE_2 on the release of TxA_2 by resident, LPS- (*A*) and cytokines- (*B*) activated KC. Liver macrophages are pre-incubated with PGE_2 (1 μM) during 1 h. Thereafter fresh medium with LPS (500 ng/ml), rat IL-1ß (5 ng/ml) or/and rat TNFa (10 ng/ml) is added and macrophages are incubated for 24 h. The amounts of TxA_2 in the medium are determined by specific ELISA as described in "Material and Methods". Data shown are means ± S.E. of at least three independent experiments.
(A) The release of TxA_2 in PGE_2-free, in resting and PGE_2-treated cells is 55.3±7.9 pmol/10^6 cells/24 h (corresponds 100%), 15±6 and 18±3 pmol/10^6 cells/24 h, respectively. $^{\#}P<0.05$ vs LPS. ***(B)*** The release of TxA_2 by PGE_2-free cells correspond 100% and is equal to 26.4±13.0 and 49.9±12.0 pmol/10^6 cells/24 h after incubation with IL-1ß and TNFa, respectively. $^{*}\boldsymbol{P}<0.01$ and $^{**}\boldsymbol{P}<0.05$ vs. cytokines.

In LPS-treated cells PGE_2 inhibits the LPS-induced mRNA expression of $cPLA_2$ (20%), COX-2 (30%) and mPGES-1 (50%) (Fig. 3.19.A) and proteins of $cPLA_2$ (50%), COX-2 (30%) and mPGES-1 (70%) (Fig. 3.19.B).

PGE_2 upregulates the expression of mRNA (Fig. 3.19.C) and proteins (Fig. 3.19.D) of IL-1β- and TNFα-induced enzymes.

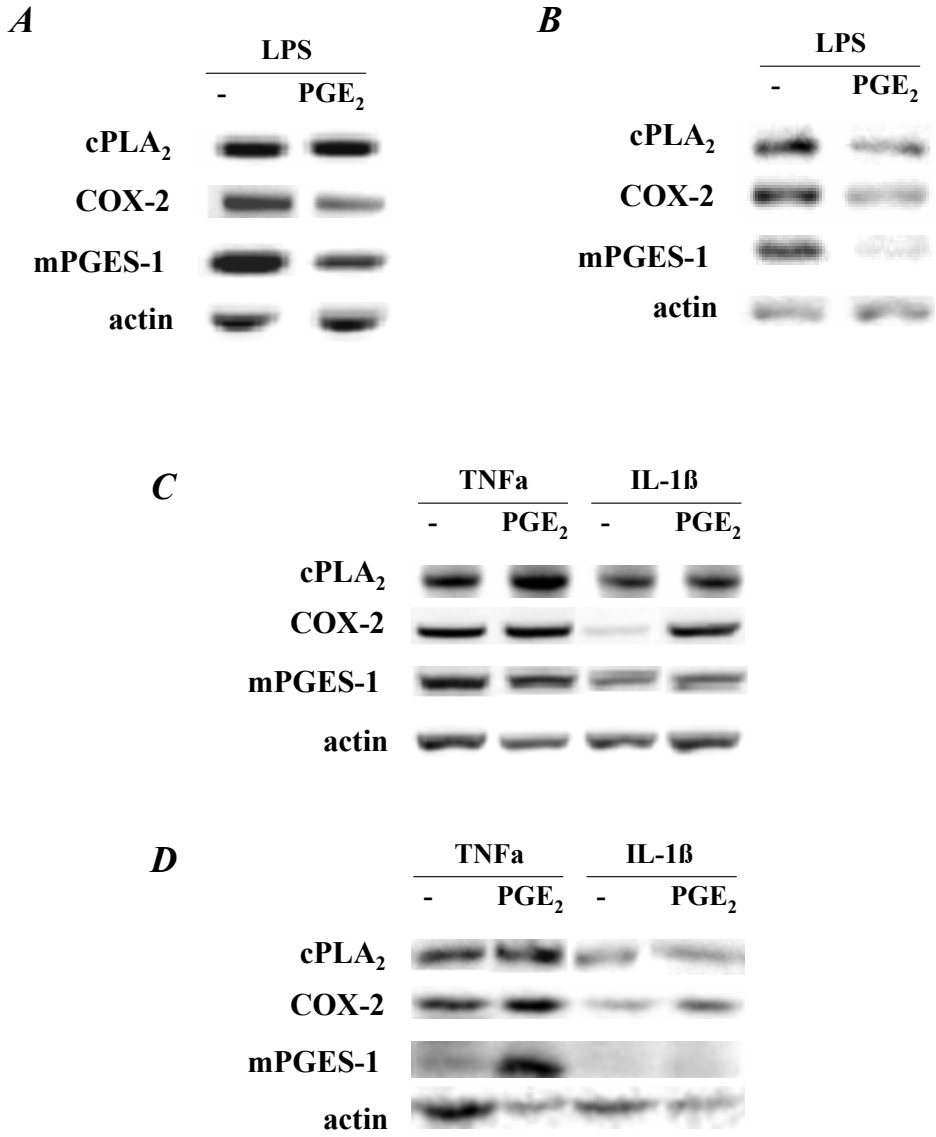

Fig. 3.19. Effect of PGE_2 on enzymes in LPS- and cytokines-activated KC. Liver macrophages are kept as described in Fig. 3.18. mRNA ***(A, C)*** and proteins ***(B, D)*** are analyzed as described in "Materials and Methods". A representative set of three ***(A, C)*** and at least two ***(C, D)*** independent experiments is shown.
(A, B) The following data are obtained after PGE_2-treatment for 24 h (no stimuli, 24 h corresponds 100): mRNA ***(A)*** $cPLA_2$ 63±31; COX-2 53±13; mPGES-1 85±25 and proteins ***(B)*** $cPLA_2$ 13±7; COX-2 70±17; mPGES-1 85±25; after PGE_2/LPS-treatment for 24 h (+LPS, 24 h corresponds 100): mRNA ***(A)*** $cPLA_2$ 81±6; COX-2 67±17; mPGES-1 47±7 and proteins ***(B)*** $cPLA_2$ 52±17; COX-2 66±9; mPGES-1 33±8.
(C, D) The following data are obtained for PGE_2/cytokines-treated cells (24 h, +IL-1ß or TNFa, respectively, corresponds 100): mRNA ***(C)*** $cPLA_2$ 100±0 and 147±11; COX-2 480±340 and 877±736; mPGES-1 185±37 and 450±166 after PGE_2 and IL-1ß/TNFa, respectively; proteins ***(D)*** $cPLA_2$ 155±32 and 181±1; COX-2 376±240 and 292±51; mPGES-1 469±72 and 730±50 after PGE_2 and IL-1ß/TNFa, respectively.

3.4. Synthesis of nitric oxide in Kupffer cells

3.4.1. Stimulation of NO release by LPS and cytokines

LPS stimulates NO release in KC (Fig. 3.20.A). The stimulation of NO release becomes visible after 2-4 h and increases linearly during the next 20 h. Moreover, the rate of NO release after LPS treatment reaches its peak already after 8 h (4.5±0.83 mol/10^6 cells/h) and remains on this level or even slightly decreases from 8 h to 24 h (4.2±1.1 pmol/10^6 cells/h).
The release of nitric oxide by LPS-activated macrophages is suppressed by dexamethasone (Fig. 3.20.C).
Cytokines are also able to modulate nitric oxide release (Fig. 3.21.). When IL-1β or TNFα are added to resident Kupffer cells (Fig. 3.21.A), a small increase of NO release is observed after 24 h of incubation (~7-8 nmol/10^6 cells, that is equal ~130%-160% vs. control cells). The combined action of both cytokines does not show a significant effect on NO release, giving ~9 nmol/10^6 cells (that is equal ~170%-190% vs. control cells).

Interleukine-10 abrogates the LPS-induced production of NO (Fig. 3.21.B). It suppresses NO release after 8 h of incubation down by 30%, after 24 h by 70%.

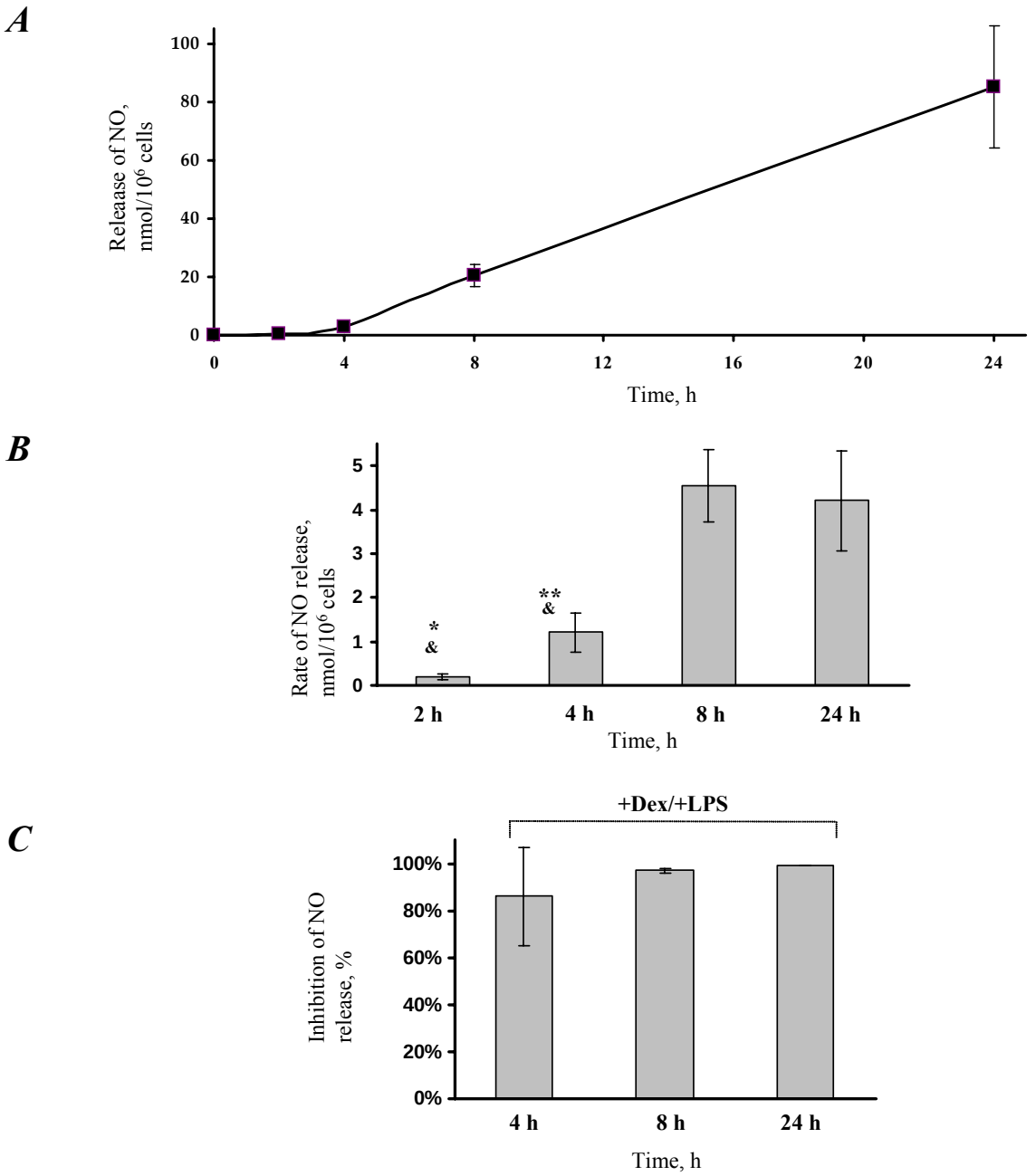

Fig 3.20. Time course study of NO-release by LPS- and dexamethasone-treated liver macrophages. (*A*) Release of nitric oxide during 24 h of LPS-stimulation. Liver macrophages (primary culture) are incubated for the indicated times in presence of LPS (500 ng/ml). The amounts of NO are determined by Griess assay as described in "Material and Methods". Values are corrected for NO formed by cells in the absence of LPS. Data shown are means ± S.E. of five independent experiments. The release of nitric oxide by resting cells is as following (in nmol/10^6 cells/4,8 or 24 h): 2.1±0.2/3.5±1.1/3.6±0.9 after 4, 8 and 24 h, respectively. **(*B*) Rates of LPS-induced nitric oxide secretion**. The rate is calculated as difference in the amount of NO formed between two time points divided by the time difference. Data shown are means ± S.E. of five independent experiments. ***P* <0.001, ****P* <0,01 (current time point vs 8 h); **&*P* <0.05 (current time point vs. 24 h),^{NS}***P*>0.05 (current time point vs. 24 h).

(*C*) Effect of dexamethasone on LPS-treated liver macrophages. Liver macrophages (primary culture) are pretreated for 1 h with dexamethasone (1 µM) or solvent. Then fresh medium with/without LPS (500 ng/ml) is added and macrophages are incubated for the indicated times. The release of NO is measured. Amounts of nitric oxide formed by resting cells are (in nmol/10^6 cells/4,8 and 24 h): 2.6±0.6/2.4±0.6/3.7±0.9 after 4, 8 and 24 h, respectively. Data shown are means ± S.E. of at least four independent experiments.

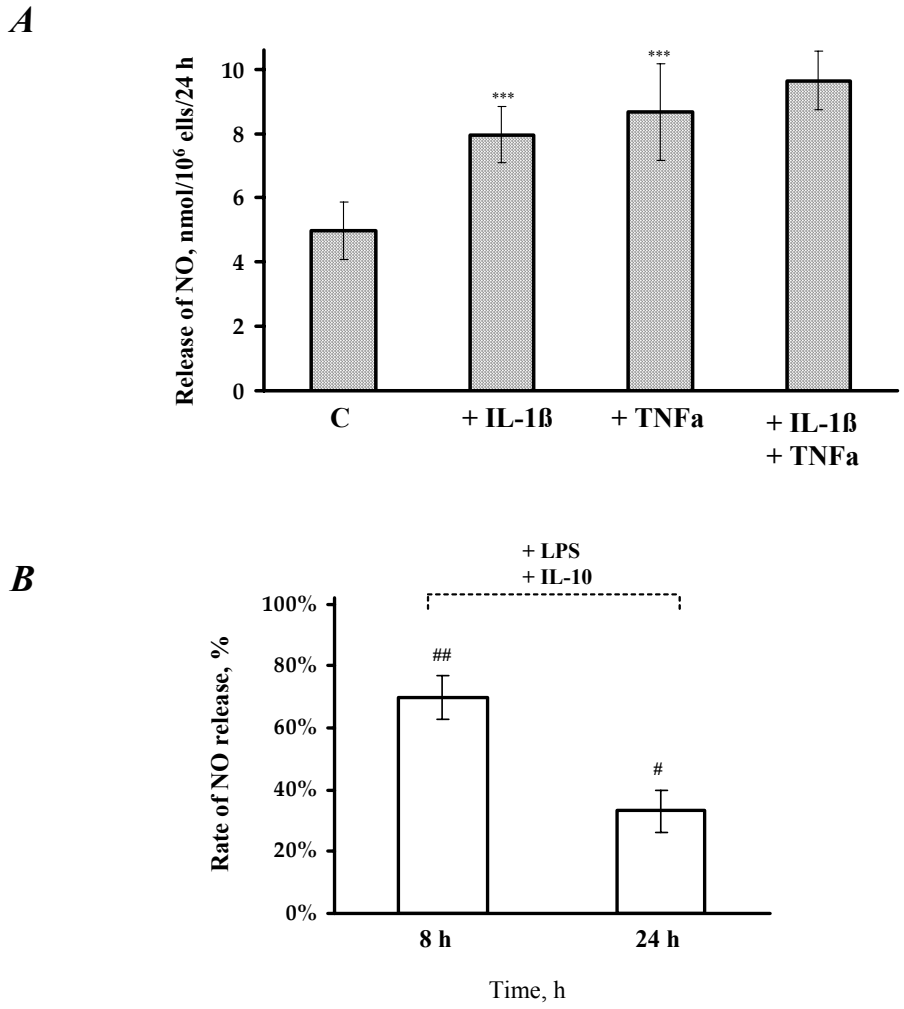

Fig. 3.21. Effect of cytokines on NO release. (A) Effect of IL-1ß and TNFa on the nitric oxide production by resident Kupffer cells. Liver macrophages are incubated for 24 h with vehicle (C), LPS (500 ng/ml), rat IL-1ß (1 ng/ml) or/and with rat TNFa (10 ng/ml, respectively**.** The amounts of nitric oxide in the medium are determined by Griess assay as described in "Material and Methods". Data shown are means ± S.E. of at least three independent experiments. $^{***}P$<0,05 vs. control cells. ***(B)* Effect of IL-10 on the nitric oxide production by LPS-activated Kupffer cells.** Liver macrophages (primary culture) are pretreated for 1 h with IL-10 (10 ng/ml) or vehicle. Then fresh medium with/without LPS (500 ng/ml) is added and macrophages are incubated for the indicated times. The amounts of nitric oxide in the medium are measured. Amounts of nitric oxide formed by IL-10-free cells are (in nmol/10^6 cells/24 h): 15.0±3.4 and 53.5±13.0 after 8 h and 24 h, respectively. Data shown are means ± S.E. of at least three independent experiments. $^{\#}P$<0.001 vs. LPS, $^{\#\#}P$<0.05 vs. LPS

3.4.2. Nitric oxide synthase isoenzymes

Three enzymes are known to be responsible for nitric oxide production: inducible (iNOS), endothelial (eNOS) and neuronal (nNOS) NO synthases (Muriel, 2000). To examine which of these enzymes are involved in NO release in KC, RT-PCR analysis of mRNA encoding NOS is performed (Fig. 3.22.A).

Control and LPS-treated Kupffer cells are examined. Rat liver endothelial cells and rat brain are used for positive control as sources of eNOS and nNOS respectively because of their property to synthesize the indicated enzymes (Alderton *et al.*, 2001).

The RT-PCR analysis of samples demonstrates that control cells do not express significant mRNA amounts encoding NO synthases. LPS induces only the expression of the iNOS mRNA (Fig. 3.22.A, B).

LPS also induces an expression of iNOS protein (Fig. 3.22.C).

The upregulation of iNOS by pro-fibrogenic cytokines (IL-1β and TNFα) is much weaker compared to LPS-treated cells (Fig. 3.22. B,C).

The downregulation of the enhanced both transcription and translation of iNOS is also studied. It is demonstrates in Fig. 3.22.D, that the expression of mRNA encoding iNOS is strongly suppressed by both anti-inflammatory agents: dexamethasone abrogates 99% of the induced iNOS transcription and after the pre-treatment of cells by IL-10, only ~1/5 of mRNA remains to be express.

Despite of the previously shown relatively high (~30%) release of nitric oxide after IL-10 treatment during 24 h of incubation, the Western blot analysis of proteins from the same sample displays only traces of iNOS in the cells lysate (Fig. 3.22.E). The synthesis of the LPS-induced iNOS seems to be completely abolished by both dexamethasone and IL-10 (~2% of proteins remains compared with LPS-treated cells).

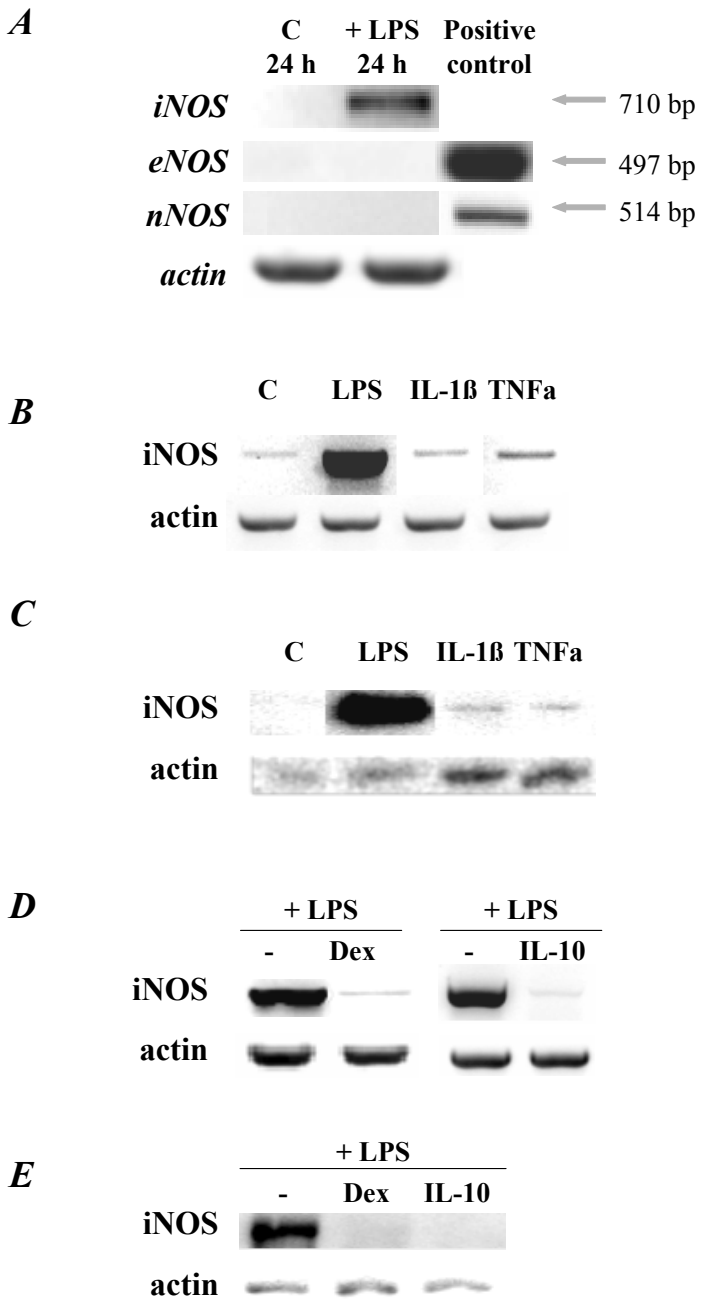

Fig. 3.22. The synthesis of NO-synthase by resident and activated liver macrophages. Liver macrophages (primary culture) are incubated for 24 h in presence of LPS (500 ng/ml), rat IL-1ß (1 ng/ml) or/and with rat TNFa (10 ng/ml) or without stimuli. mRNA (***A, B***) and proteins (***C***) of NO-synthases are analyzed as described in "Materials and Methods". A representative set of three independent experiments is shown. ***(B)*** The expression of enzyme in unstimulated cells corresponds 100, in LPS-, IL-1ß- and TNFa-treated cells is 3793±576, 220±81 and 243±120, respectively (n=3); ***(C)*** The synthesis of enzyme in unstimulated cells corresponds 100, in LPS-, IL-1ß- and TNFa-treated cells is 12024±3488, 340±70 and 335±135, respectively (n=2). ***(D, E)*** **Regulation of iNOS by dexamethasone and IL-10 in LPS-activated macrophages.** Cells are pre-treated for with IL-10 (10 ng/ml) and dexamethasone (Dex, 1 μM) for 1 h. Then fresh medium with LPS (+LPS; 500 ng/ml) or without (-LPS) is added and macrophages are incubated for 24 h. mRNA (***D***) or proteins (***E***) are analyzed. A representative set of three independent experiments is shown. (***D***) The expression of iNOS in IL-10/LPS- and Dex/LPS- treated cells is 22±10 and 2±0, respectively (24 h, +LPS corresponds 100); ***(E)*** **t**he synthesis of iNOS in IL-10/LPS- and Dex/LPS- treated cells **is** 2±1 and 2±2, respectively (24 h, +LPS corresponds 100).

3.5. Interaction of nitric oxide and prostanoid pathways in Kupffer cells

3.5.1. Effect of NO on prostanoid synthesis

3.5.1.1. Effect of NO-donor on prostanoid synthesis

The effect of exogenous nitric oxide on prostanoid synthesis is assessed with the NO-donor S-Nitroso-N-acetylpenicillamine (SNAP), which delivers nitric oxide in aqueous buffers ($t_{½}$ = 10 h) (Fig. 3.23.A). It is found, that SNAP leads to the formation of NO in a concentration-dependent way and to a release of PGE_2 (Fig. 3.23.A).

Simultaneously, SNAP induces in a concentration-dependent way the expression of $cPLA_2$, COX-1 and mPGES-1 (Fig. 3.23.A).

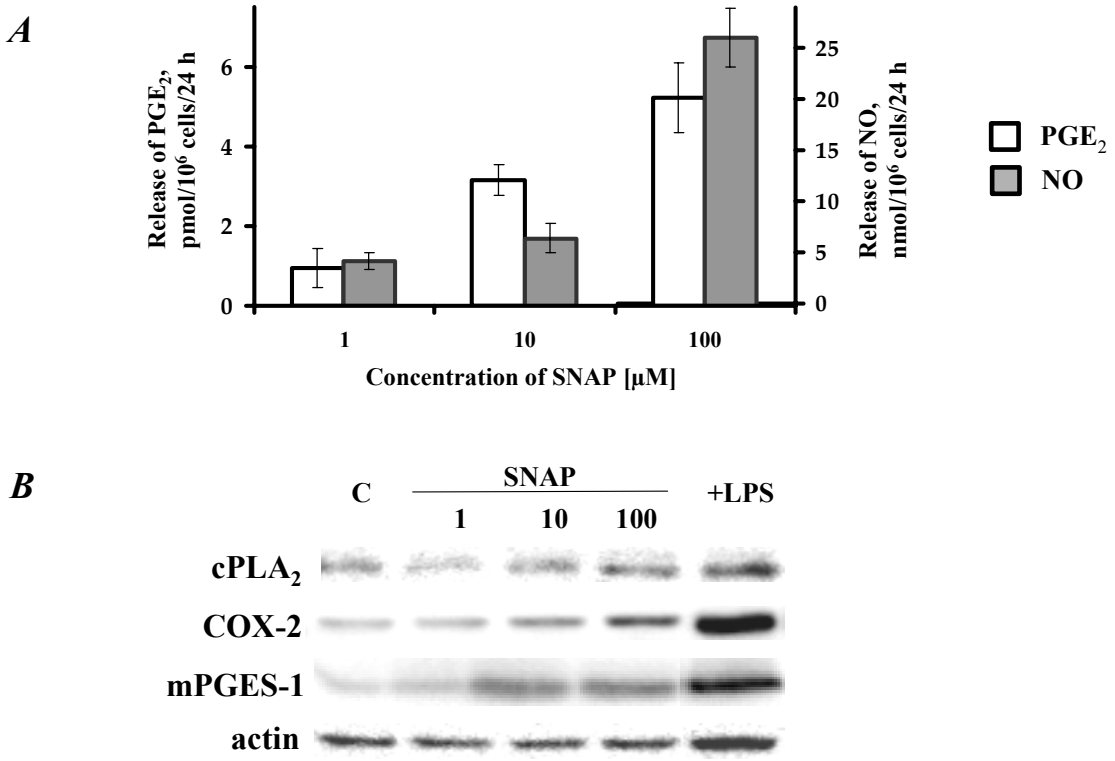

Fig. 3.23. Effect of SNAP and endogenous NO on the PGE_2 release and enzymes. Liver macrophages are incubated with SNAP (S-Nitroso-N-acetylpenicillamine; 1, 10 or 100 μM) or vehicle (C) for 24 h. ***(A)*** The amounts of nitric oxide and PGE_2 in the medium are determined by Griess assay and specific ELISA, respectively (the values are corrected for NO and PGE_2 formed in the absence of stimuli). Data shown are means of at least three independent experiments ± S.E. The release of PGE_2 in SNAP(1 μM)/LPS-treated cells is equal to 81% ±5%, when the release of PGE_2 in LPS-treated cells is 18.7±3.9 pmol/10^6 cells/24 h.
(B) Proteins are analyzed as described in "Materials and Methods". A representative set of at least two independent experiments is shown. The synthesis of enzymes after tretament for 24 h with 10 μM and 100 μM of SNAP are (24 h, no stimuli, corresponds 100): $cPLA_2$ 156±2 / 187±43; COX-2 129±27 / 170±26; mPGES-1 251±61 / 282±87, respectively.

3.5.1.2. Blockade of endogenous nitric oxide secretion

The downregulation of endogenous nitric oxide release by LPS-activated Kupffer cells is studied by two NOS inhibitors: L-NMMA (N^G-Monomethyl-L-arginine monoacetate) which competitively inhibits three isoforms of NOS and by L-NIL (L-N^6-(1-Iminoethyl) lysine) which acts as a selective inhibitor of iNOS. Kupffer cells, treated with LPS and inhibitors showed a strong reduction of NO release (Table 3.3.). The specific inhibition of iNOS by L-NIL is more effective as the non-specific inhibition by L-NMMA.

L-NIL and L-NMMA lead to a weak inhibition of PGE_2-release.

L-NIL and L-NMMA have no effect on the expression of $cPLA_2$, COX-2 and mPGES-1 (Fig. 3.24.).

	% of inhibition	
	NO	**PGE_2**
L-NIL	**69 ± 4***	**15 ± 7****
L-NMMA	**59 ± 8***	**15 ± 5****

Table 3.3. The inhibition of NO and PGE_2 release by L-NIL and L-NMMA. Liver macrophages (primary culture) are pretreated for 15 min with L-NMMA (N^G-Monomethyl-L-arginine monoacetate, 10 µg/ml), L-NIL (L-N^6-(1-Iminoethyl) lysine; 10 µM) or solvent. Then fresh medium with/without LPS (500 ng/ml) is added and macrophages are incubated for the indicated times. The amounts of NO and PGE_2 in cell media are determined by Griess assay or ELISA, respectively, as described in "Material and Methods". Data shown are means ± S.E of at least three independent experiments. The release of NO and PGE_2 in LPS-pretreated cells is 95±15 nmol/10^6 cells and 23±4 pmol/10^6 cells, respectively. $^*\boldsymbol{P}$<0.01, $^*\boldsymbol{P}$ <0.055.

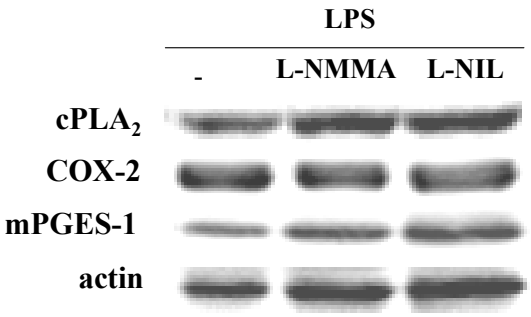

Fig. 3.24. Effect of L-NMMA and L-NIL on the enzyme synthesis. Cells are kept as described in the Table 3.3. Proteins are analyzed as described in "Material and Methods". A representative set of three independent experiments is shown. The synthesis of enzymes in L-NMMA/LPS- and L-NIL/LPS-treated cells is (24 h, +LPS corresponds 100): $cPLA_2$ 81±4 / 99±5; COX-2 104±3 / 115±13; mPGES-1 108±8 / 132±9, respectively.

3.5.2. Effect of PGE_2 on NO synthesis

3.5.2.1. Effect of exogenously added PGE_2 on nitric oxide synthesis

PGE_2 affects differently the NO synthesis in liver KC (Fig. 3.25., Fig. 3.26.):

1) PGE_2, added exogenously to the resident cells has no effect on NO release (data not shown);

2) PGE_2, added exogenously to LPS-treated KC inhibits the NO release to 60% (Fig. 3.25.A). The exogenous PGE_2 downregulates iNOS mRNA and proteins by more than 90% (Fig. 3.26.);

3) PGE_2, added exogenously to cytokines-treated KC, increases NO release by 140% (IL-1β), 400% (TNFα) and about 400% (IL-1β/TNFα).

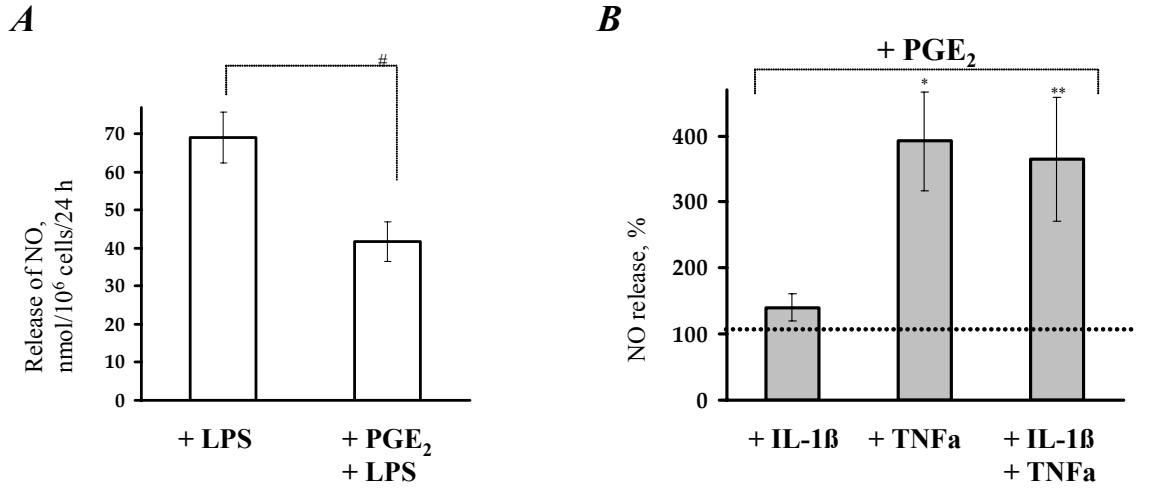

Fig. 3.25. Effect of PGE_2 on the release of NO by LPS-*(A)* and cytokines-*(B)* activated KC. Liver macrophages are pre-incubated with PGE_2 (1 μM) during 1 h. Thereafter fresh medium with LPS (500 ng/ml), rat IL-1ß (5 ng/ml) or/and rat TNFa (10 ng/ml) is added and macrophages are incubated for 24 h. The amounts of nitric oxide are determined by Griess assay as described in "Material and Methods". Data shown are means ± S.E. of at least three independent experiments. ***(A)*** $^{\#}P<0.05$ vs. LPS. The release of nitric oxide in resting and PGE_2-treated KC is 1.3±0.2 and 1.6±0.2 nmol/10^6 cells/24 h, respectively. ***(B)*** 100% corresponds the cytokines-mediated NO release in absence of PGE_2 (in nmol/10^6 cells/24 h): 10.1±1.4, 7.8±1.4 and 9.2±2.0 after incubation with IL-1ß, TNFa and IL-1ß+TNFa, respectively.****P***<0.01 and *****P***<0.05 vs. cytokines.

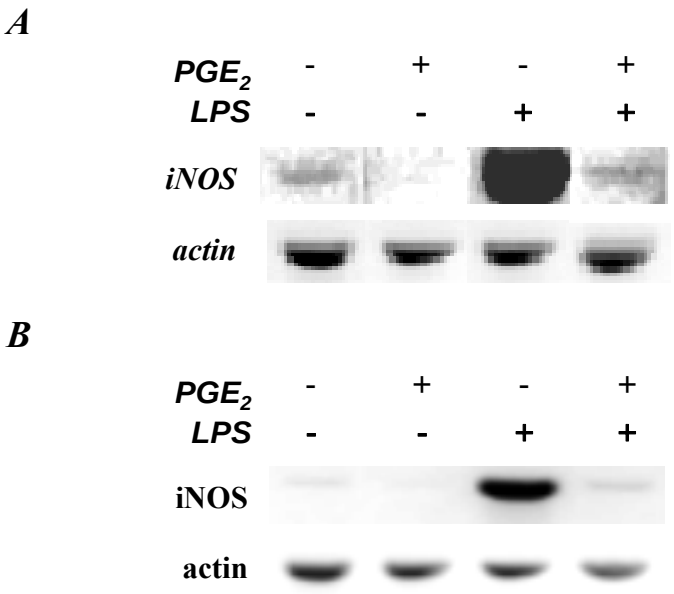

Fig. 3.26. Effect of exogenously added PGE_2 on inducible NO synthase. Liver macrophages are pre-incubated with PGE_2 (1 μM) during 1 h. Thereafter fresh medium with/without LPS (500 ng/ml) is added and macrophages are incubated for 24 h. mRNA **(*A*)** or proteins **(*B*)** are analyzed as described in "Materials and Methods". A representative set of three independent experiments is shown. The following data of densitometric analysis are obtained (24 h, +LPS corresponds 100): mRNA - 9±5, proteins - 4±2 after incubation with PGE_2 and LPS for 24 h.

3.5.2.2. Effect of cell-derived PGE_2 on nitric oxide production

Indomethacin is a member of the non-steroidal anti-inflammatory drug family, capable to inhibit both COX-1 and COX-2. PGE_2 synthesis in LPS-activated liver macrophages is almost completely inhibited by indomethacin (Fig. 3.27.A).

Indomethacin suppresses about 40% of NO release.

It has no effect on the expression of iNOS, $cPLA_2$, COX-2 and mPGES-1 (Fig. 3.27.B).

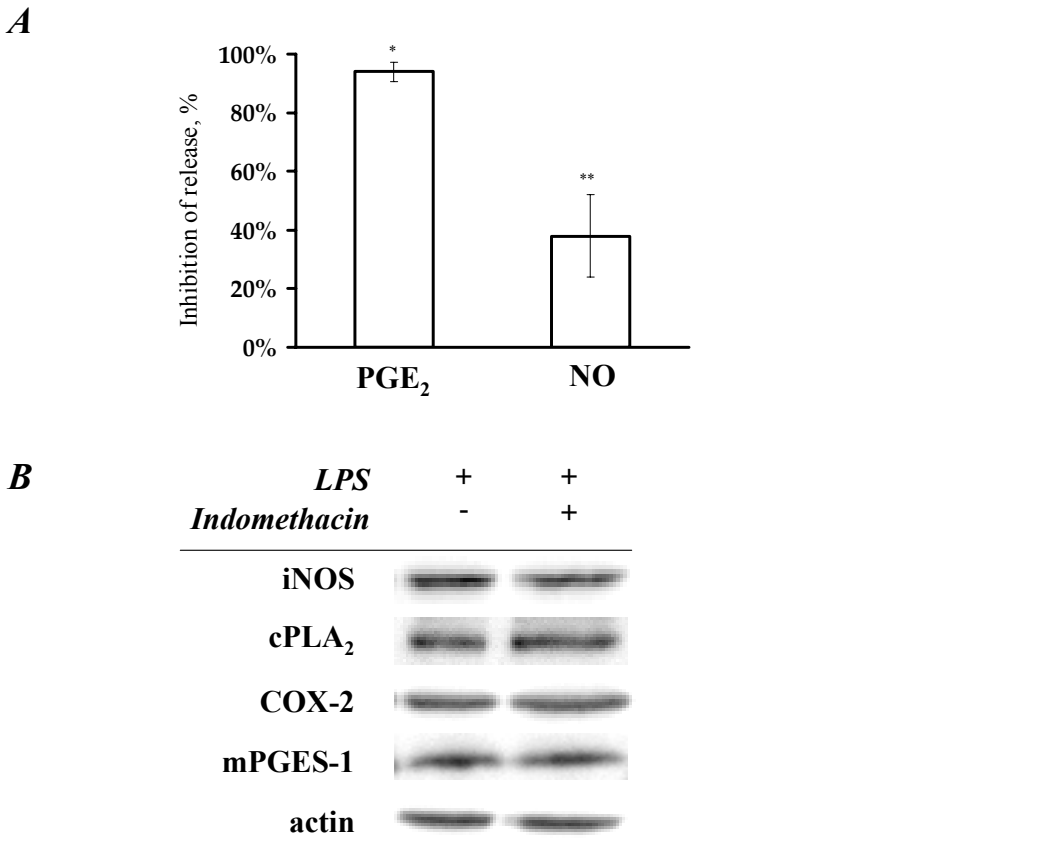

Fig. 3.27. Effect of indomethacin on the release of both PGE_2 and NO and on the synthesis of corresponding enzymes. Liver macrophages (primary culture) are pretreated for 1 h with indomethacin (1 μM) or solvent. Then fresh medium with/without LPS (500 ng/ml) is added and macrophages are incubated for 24 h. (***A***) The amounts of PGE_2 and NO in the supernatants are determined by specific ELISA or by Griess assay, respectively, as described in "Material and Methods". Data shown are means ± S.E of eight independent experiments. The release of PGE_2 and NO LPS-treated cells is 20±1 pmol/10^6 cells and 74±15 nmol/10^6 cells. ****P***<0.001, *****P*** <0.05 vs. LPS-treated macrophages. **(*B*)** Proteins are analyzed as described in "Materials and Methods". A representative set of three independent experiments is shown. The synthesis of indicated enzymes in indomethacin/LPS-treated cells is (24 h, +LPS corresponds 100): iNOS 96±5; $cPLA_2$ 89±6; COX-2 106±11; mPGES-1 94±14.

4. Discussion

This study focuses on the prostanoid and nitric oxide production by rat liver macrophages, on the interaction of their synthesis pathways and on the regulation of their synthesis by pro- and ant-fibrogenic cytokines.

4.1. Study of prostanoid synthesis

Prostanoids play significant role in the organism and in the liver in particular. PGE_2 release is strongly enhanced by pro-inflammatory stimuli (Dieter *et al.*, 1995; Ambs *et al.*, 1995; Dieter *et al.*, 2002; Dieter *et al.*, 2000) and plays an important role in the regulation of liver (patho)physiology **(**Dieter *et al.*, 2001**)**. Thromboxane A_2 is a potent modulator, involved in pathophysiology and is released from Kupffer cells (Decker, 1990).

The present study demonstrates that rat liver macrophages release an enhanced amount of PGE_2 and TxA_2 during the prolonged incubation with LPS.
In recent studies there has been shown, that lipopolysacharide – a potent bacterial endotoxin from the outer membrane of gram-negative bacteria - provokes a strong inflammatory response by macrophages (Dieter *et al.*, 1995), in particular by Kupffer cells (Ambs *et al.*, 1995; Thoren *et al.*, 2000). In contrast to other stimuli (e.g. zymosan, phobol ester, Ca^{2+} ionophore) which lead to an immediate release of mediators in the cultivation medium (Ambs *et al.*, 1995), the response of macrophages on LPS activation occurs often after a lag phase of several hours.

The release of TxA_2 is fast and becomes considerable already after 2 h of incubation; thereafter the rate of synthesis declines. In opposite, PGE_2 release shows a “lag-phase” and becomes measurable after 4 h of incubation. Thereafter, it displays an almost linear dependence on the time of incubation, increasing gradually during the next 20 h.

Dexamethasone downregulates the PGE_2 and TxA_2 release, like in human A549 cell line (Thoren *et al.*, 2000), in rat peritoneal macrophages (Murakami *et al.*, 2000) or in human rheumatoid synoviocytes (Stichtenoth *et al.*, 2001).
The distinction in the release kinetics of both TxA_2 and PGE_2, together with earlier findings (Dieter *et al.*, 1989) suggest different regulatory mechanisms in their synthesis pathways. The synthesis of prostanoids goes through the three consecutive stages, including i) release of AA by $cPLA_2$, ii) its successive conversion into PG G_2/H_2 by means of cyclooxygenase isoenzymes and iii) the subsequent synthesis of prostanoids from PG G_2/H_2 through the action of final prostanoid synthases. Each step of the prostanoid synthesis is an object for the regulation by pro- and anti-inflammatorFy agents.

The regulation can occur on the enzymes activities as well as on transcription and translation level. Here is demonstrated that LPS induces a coordinate expression of $cPLA_2$, COX-2 and mPGES-1, while COX-1, cPGES, mPGES-2 and TxAS remain unchanged. In detail:

1) $cPLA_2$: $cPLA_2$ is expressed at low levels in resident KC. $cPLA_2$ protein is localized predominantly in the membrane fraction. LPS induces also a phosphorylation of $cPLA_2$. These studies confirm earlier findings (Ambs *et al.*, 1995) that $cPLA_2$ undergoes induction and posttranslational modification through phosphorylation when KC are exposed to LPS;

2) Cyclooxygenases: COX-1 is expressed in resident KC. COX-1 does not undergo significant changes on both transcriptional and translational level by LPS. COX-2 is detected in resident KC at very low levels. LPS induces an enhanced mRNA expression with a maximum at 4 h. COX-2 protein is synthesized in a linear mode reaching a maximum after 24 h of incubation. Both, mRNA encoding COX-2 and COX-2 protein are completely suppressed by dexamethasone. Both, COX-1 and COX–2 are localized mainly in the membrane fraction.

3) Final prostaglandin synthases:
   - ***mPGES-1:*** is expressed at very low level in resident cells; LPS induces a high induction at mRNA and protein level; present in microsomal fraction;
   - ***mPGES-2:*** is expressed in resident cells; LPS induces a weak induction at mRNA and a prolonged induction at translation level; present in microsomal and partially in cytosolic fraction;

- ***cPGES:*** is expressed in resident cells; LPS does not alter expression at mRNA and protein level; present in cytosolic fraction;
- ***TxAS:*** like cPGES; present in microsomal and (partially) in cytosolic fraction. TxAS activity is not changed by LPS.

PGE_2 synthesis and release might be regulated by means of activation/suppression of each steps i) $cPLA_2$, COX-2, mPGES-1 and mPGES-2 or by means of posttranslational modifications of cPGES (Tanioka *et al.*, 2003; Kobayashi *et al.*, 2004). It has been shown that the LPS-induced mPGES expression is regulated through a Toll-like receptor 4 (TLR4)/MyD88-dependent pathway and depended upon the NF-IL6 transcription factor (Uematsu *et al.*, 2002); stimuli-induced expression of the mouse and human mPGES has been found to require the inducible nuclear protein Egr-1 (Early Growth Response–1) (Naraba *et al.*, 2002).

Another aspect of regulation is the question how much each distinct enzyme contributes to PGE_2 production. These questions on the coupling between the different PGES and COX enzymes are widely discussed (Dieter *et al.*, 2000; Lazarus *et al.*, 2002a; Naraba *et al.*, 1998; Tanioka *et al.*, 2000; Murakami *et al.*, 2000; Thoren *et al.*, 2000; Stichtenoth *et al.*, 2001). A previous study using specific COX-1 and COX-2 inhibitors demonstrates that in Kupffer cells PGE_2 is more effectively coupled to COX-1 (Dieter *et al.*, 2000). Other studies have been recently performed with cotransfection of cPGES (Tanioka *et al.*, 2000) and mPGES-1 (Murakami *et al.*, 2000) showing a coupling between COX-2 and mPGES-1, and COX-1 and cPGES. Also a colocalization of mPGES-1 and COX-2 in mice has been detected by Lazarus *et al.*, 2002b. In contrast, another study demonstrates that mPGES-1 is coupled to COX-1 only in an immediate activation of $cPLA_2$, but in a delayed response mPGES-1 is coupled to COX-2 (Murakami *et al.*, 2000). These studies suggest that the coupling process might depend on the tissue and/or the stimulus.

Here, using specific COX-1 and COX-2 inhibitors it is shown that in general COX-1 is more involved in release of TxA_2, whereas COX-2 mediates the release of PGE_2 (Fig. 4.1.). However, the participation of the COX isoenzymes is also dependent on the time of the release: in TxA_2 release COX-2 becomes more significant at earlier time point, in PGE_2 release COX-1 becomes more significant at later time point.

The coupling to the final prostanoid synthases can only be hypothesized at present: in the early release, the COX isoenzymes couple to the pre-existing cPGES, mPGES-2 and TxAS. The level of the induced mPGES-1 at later time points have to be further investigated.

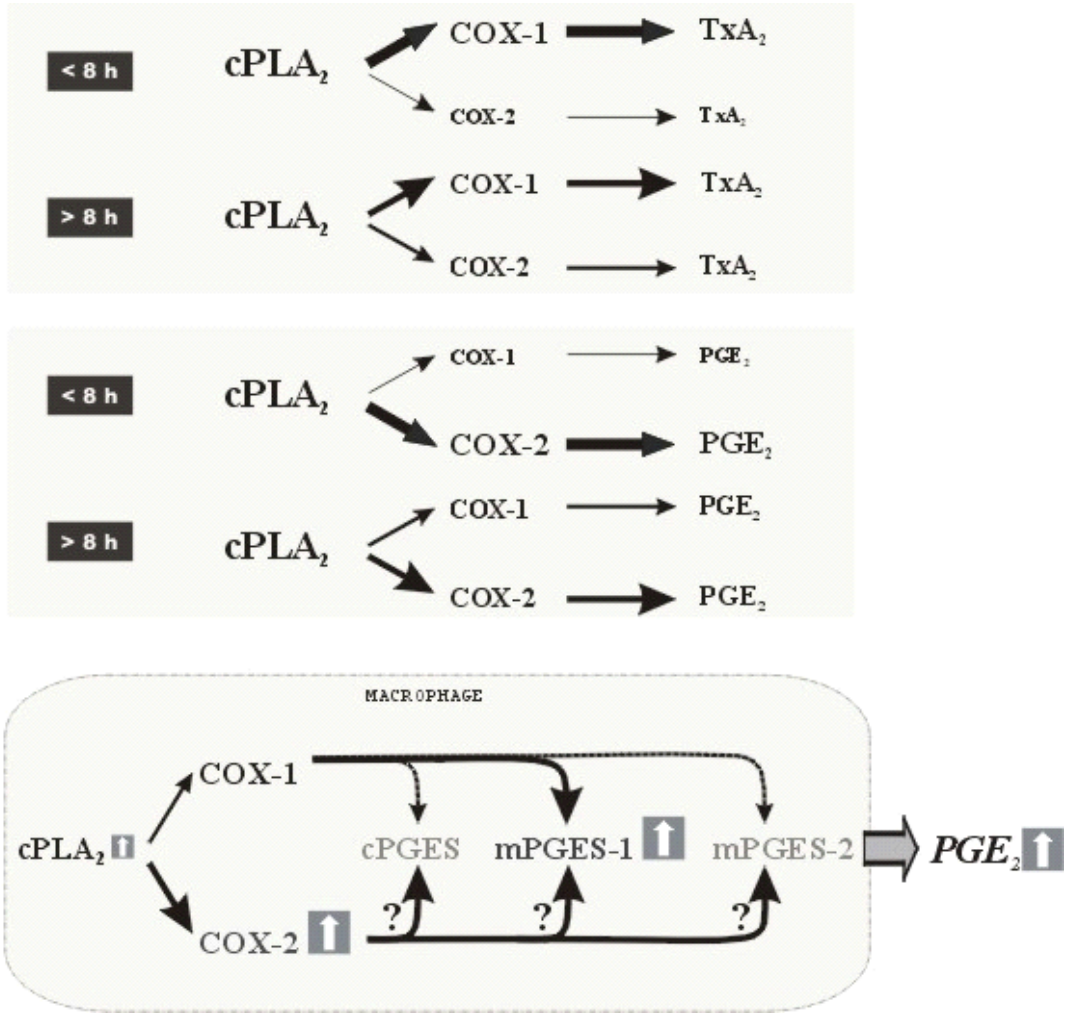

Fig. 4.1. Tentative scheme for LPS-induced release of PGE_2 and TxA_2 in liver macrophages.

4.2. Effect of cytokines on resident and LPS-activated cells

4.2.1. Pro-fibrogenic cytokines activate the synthesis of prostanoids

Numerous studies report that under inflammatory conditions KC release cytokines of the acute phase response, such as IL-1β, TNFα and IL-6 (Fig. 4.2.) (Decker, 1990; Treffkorn *et al.*, 2004; Karck *et al.*, 1988; Oguro *et al.*, 2002; Nunez *et al.*, 2005). Other liver cells are also capable to release pro-fibrogenic cytokines (Malik *et al.*, 2002; Olinga *et al.*, 2001).

The present study shows that both IL-1β and TNFα induce the synthesis of PGE_2 and TxA_2. The cytokines exert a synergistic effect on PGE_2, but not on a TxA_2 release. The cytokine-mediated regulation of prostanoid synthesis occurs on the level of mRNA and protein of $cPLA_2$, COX-2 and mPGES-1.

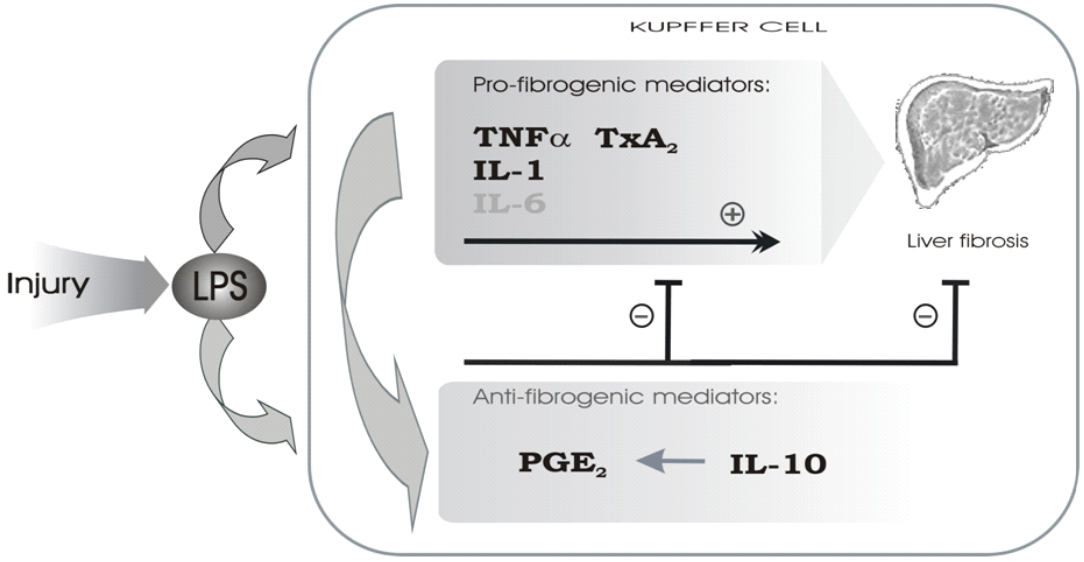

Fig. 4.2. Tentative schema of cytokine and prostanoid interaction

In contrast, IL-6 does not have a effect on the release of prostanoids. IL-6 has been designated to be a proliferative factor for rat HSC and hepatocytes (Malik *et al.*, 2002).

4.2.2. Anti-fibrogenic cytokines

IL-10 is widely produced by liver cells *in vivo* and *in vitro,* protecting the liver against injury. For instance, IL-10 has been reported to suppress the hepatic injury in mice (Ju *et al.*, 2002; Kahlke *et al.*, 2002; Thompson *et al.*, 1998), to modulate TNFa action *in vitro* on rat hepatic macrophages (Tran-Thi *et al.*, 1995) and human choriodecidua (Sato *et al.*, 2003). A regulatory effect of IL-10 on prostanoid production has been previously described in an endotoxine-mediated mice model (Berg *et al.*, 2001), in human monocytes (Niiro *et al.*, 1995) and neutrophils (Nagano *et al.*, 2002).

Here it is shown that LPS-induced release of PGE_2 and TxA_2 is suppressed by IL-10 on the level of mRNA and protein of $cPLA_2$, COX-2 and mPGES-1. Thus, IL-10 abrogates not only the LPS-induced increase of $cPLA_2$ and COX-2 expression (Fernandez-Morata *et al.*, 2000; Nagano *et al.*, 2002; Berg *et al.*, 2001; Niiro *et al.*, 1995). Our result is consistent with the recent studies demonstrating a hepato-protective properties of IL-10 (Olinga *et al.*, 2001; Nelson *et al.*, 2003; Pinzani *et al.*, 2001; Kojima *et al.*, 2003).

4.3. Production of nitric oxide by rat liver macrophages

The role of nitric oxide in the liver is dualistic, but it is clear that it regulates a number of (patho)physiological processes in the liver. For instance, NO has been shown to be harmful or protective for hepatocytes (Kawada N., 1999), to affect the transcription of genes that modulate post-injury proliferation or cell death (Horton *et al.*, 1994), to nitrosylate cellular enzymes (Liu *et al.*, 2002) and to inhibit mitochondrial respiratory chain enzymes and glyconeogenesis (Horton *et al.*, 1994). Furthermore, NO has been shown to play an essential role in the initiation of the inflammatory response in the liver (Liu *et al.*, 2002) and to modulate the hepatocyte's response to injury. Nitric oxide may also prevent the necrotic cell death following endotoxemia-induced liver damage (Ou *et al.*, 1997). In hepatocyte cultures nitric oxide has been shown to inhibit total protein synthesis and bile canalicular contraction (Gaillard *et al.*, 1991) and to prevent apoptosis (Harbrecht *et al.*, 1995).

The synthesis of NO in rat Kupffer cells has been demonstrated earlier (Decker, 1990; Stadler *et al.*, 1993; Gaillard *et al.*, 1991).

Here it is shown that treatment of rat liver macrophages with LPS leads to the expression of iNOS and to the release of NO.

Furthermore, we provide evidence that IL-1β and TNFα induce a small release of NO and exert a small induction of iNOS mRNA and protein expression.

In contrast, IL-10 strongly abrogates the LPS-mediated NO release and mRNA and protein expression of iNOS in rat liver macrophages.

4.4. Interaction of prostanoids and nitric oxide biosynthesis pathways

To examine the effect of exogenous nitric oxide on the PGE_2 release and its synthesis pathways, NO-donor SNAP has been used in this study. It is shown that endogenous NO induces synthesis of PGE_2, expression of $cPLA_2$, COX-2 and mPGES-1.

Furthermore, the effect of a suppression of endogenous nitric oxide release by NOS inhibitors (L-NMMA, L-NIL) is examined. Both inhibitors lead to a small suppression of PGE_2 release, but do not affect the enzyme expression.

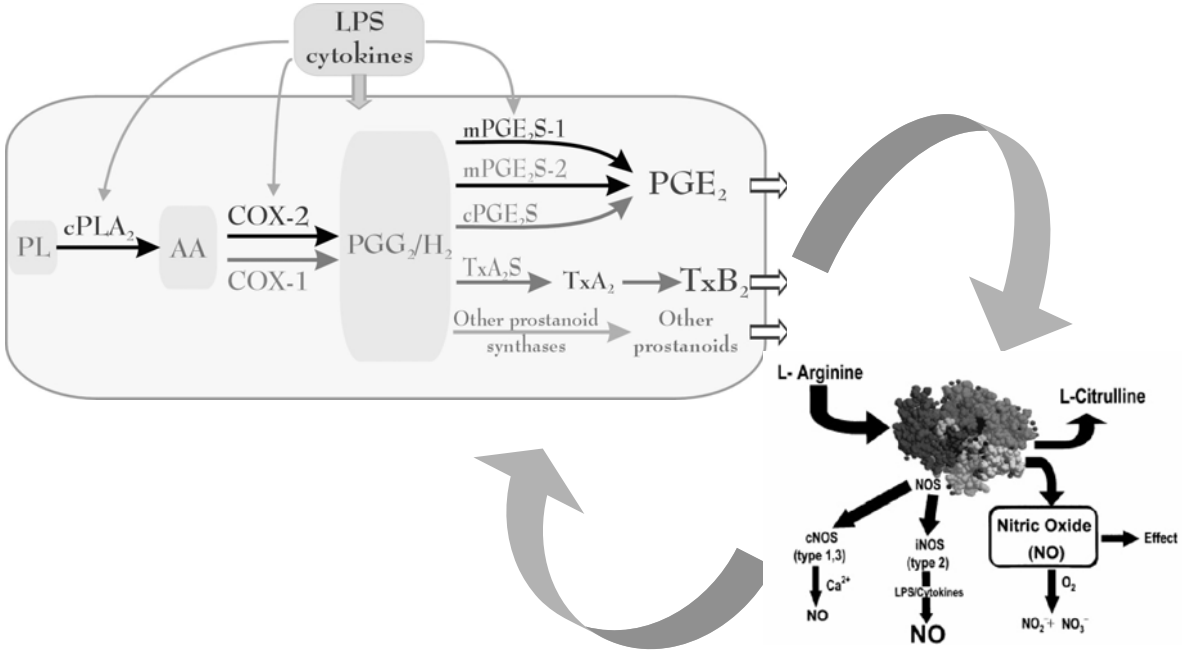

Fig. 4.3. Interaction between prostanoid and NO release system in rat Kupffer cells.
(Nitric oxide synthesis has been demonstrated in J Appl Toxicol. 2000;20(3):189-95.)

In literature, opposite effects of PGE_2 and NO release have been described (Harbrecht *et al.*, 1995; Gaillard *et al.*, 1991).

Here it is shown that:

- PGE_2 has no effect on NO release in resident cells;
- PGE_2 inhibits NO release and iNOS mRNA and protein in LPS-simulated cells;
- PGE_2 induces a small (IL-1β) and a strong (TNFα) increase in NO release;

Inhibition of PGE_2 release by indomethacin leads to an inhibition of the LPS-mediated nitric oxide production; but does not affect the expression of iNOS.

These data are interpreted (Fig. 4.3.) as follows:

- Nitric oxide is a potent regulator of PGE_2 release;
- PGE_2 is a potent regulator of NO release;

Our data are consistent with previous studies in other animal models (Watkins *et al.*, 1997; Fermor *et al.*, 2002; Gallo *et al.*, 2002; Perkins *et al.*, 1999).

4.5. Prostanoids are important regulators of the liver (patho)physiology

Prostanoids play an important regulatory role in the liver in normal and inflammatory conditions (Dieter *et al.*, 1999; Dieter *et al.*, 2001). They exert an effect on the production of other macrophage-derived mediators, like cytokines and NO and they regulate prostanoid release in an autocrine way.

In detail:

- LPS induce a strong release of TxA_2;
- TNFα upregulates TxA_2 release whereas IL-1β shows only a small response;
- LPS-induced expression of $cPLA_2$, COX-2 and mPGES-1 is strongly inhibited by PGE_2;
- TNFα-induced expression of enzymes is upregulated by PGE_2.

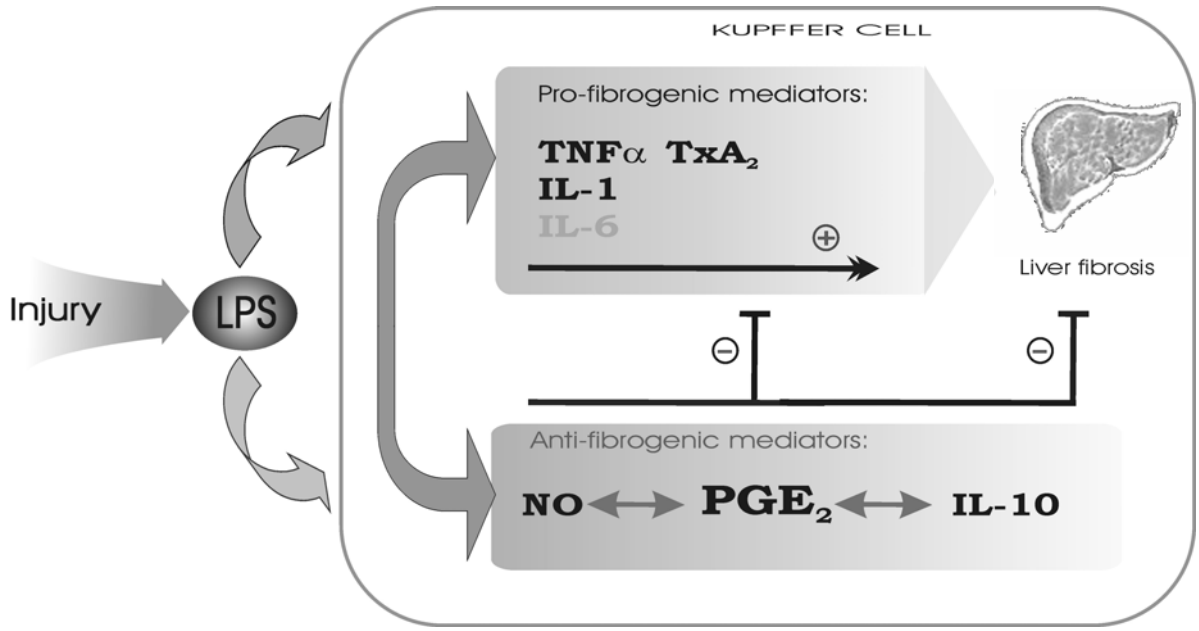

Fig. 4.4. Schematic presentation of prostanoids, cytokines and nitric oxide network in liver macrophages during fibrogenesis

These data confirm preliminarily studies on the action of PGE_2 in liver macrophages (Fig. 4.4.).

PGE_2 exerts action as an anti-fibrogenic mediator by modulation of:

- pro-fibrogenic mediators as TNFα (Shinomiya *et al.*, 2001), IL-1β (Goss et al., 1993), TxA_2;
- anti-fibrogenic mediators as IL-10 (Goss et al., 1993) and NO;

and by that inhibits the process of liver fibrogenesis.

SUMMARY

Resident liver macrophages (Kupffer cells) play an important role in liver injury and liver fibrogenesis. They are the main producers of inflammatory mediators within the liver and are capable to release pro-fibrogenic mediators like IL-1β, TNFα, IL-6, TxA_2 and anti-fibrogenic mediators like PGE_2, NO and IL-10.

Furthermore, liver macrophages are the high-capacity source of prostanoids (PGE_2, TxA_2) in the liver. Prostanoid synthesis consists of several steps including arachidonic acid release by phospholipase ($cPLA_2$), the conversion of AA in prostaglandin G_2/H_2 by cyclooxygenases (COX-1 and COX-2) and the synthesis of prostanoids by different final prostanoid synthases.

LPS and dexamethasone have opposite effects on the release of PGE_2 and TxA_2, and on the expression of enzymes involved in the synthesis of these two mediators; LPS upregulates the release of PGE_2 and TxA_2 and the expression of COX-2 and mPGES-1; dexamethasone suppresses the induction.

COX-1 and COX-2 contribute differently to the LPS-induced release of PGE_2 and TxA_2 in a time-dependent way.

LPS also induces an expression of iNOS and a release of NO, dexamethasone suppresses the upregulation.

The cytokines, IL-1β and TNFα, induce a release of PGE_2, TxA_2 and NO and an expression of $cPLA_2$, COX-2, mPGES-1 and iNOS. IL-6 has no effect. IL-10 suppresses the LPS-mediated release of PGE_2 and NO and the expression of correspondent enzymes.

Endogenous NO leads to an enhanced release of PGE_2 and expression of $cPLA_2$, COX-2 and mPGES-1. Inhibition of NO release leads to a decreased release of PGE_2. Inhibition of PGE_2 release leads to a decreased release of NO.

Exogenously added PGE_2 reduces the LPS-induced release of NO and TxA_2 but augments the TNFα- and IL-1β-induced release of NO and TxA_2.

These results demonstrate a crosstalk between the prostanoids, cytokines and NO in Kupffer cells which might be very important in the development of liver fibrosis.

REFERENCES

Albanis, E. and Friedman, S. L. (2001). Hepatic fibrosis. Pathogenesis and principles of therapy. *Clin.Liver Dis.*, **5**, 315-3vi.

Alderton, W. K., Cooper, C. E. and Knowles, R. G. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochem.J.*, **357**, 593-615.

Amber, I. J., Hibbs, J. B., Jr., Taintor, R. R. and Vavrin, Z. (1988). Cytokines induce an L-arginine-dependent effector system in nonmacrophage cells. *J.Leukoc.Biol.*, **44**, 58-65.

Ambs, P., Baccarini, M., Fitzke, E. and Dieter, P. (1995). Role of cytosolic phospholipase A2 in arachidonic acid release of rat-liver macrophages: regulation by Ca2+ and phosphorylation. *Biochem.J.*, **311 (Pt 1)**, 189-195.

Appleton, I., Tomlinson, A. and Willoughby, D. A. (1996). Induction of cyclo-oxygenase and nitric oxide synthase in inflammation. *Adv.Pharmacol.*, **35**, 27-78.

I. Arias, Popper, H., Scachter, D. and Shafritz, D. A. (1982). *The Liver: Biology and Pathobiology*. Raven Press, New York. 493-520.

Armbrust, T. and Ramadori, G. (1996). Functional characterization of two different Kupffer cell populations of normal rat liver. *J.Hepatol.*, **25**, 518-528.

Bautista, A. P. (2000). Impact of alcohol on the ability of Kupffer cells to produce chemokines and its role in alcoholic liver disease. *J.Gastroenterol.Hepatol.*, **15**, 349-356.

Beckh, K., Kneip, S. and Arnold, R. (1994). Direct regulation of bile secretion by prostaglandins in perfused rat liver. *Hepatology*, **19**, 1208-1213.

Belanger, M., Desjardins, P., Chatauret, N. and Butterworth, R. F. (2002). Loss of expression of glial fibrillary acidic protein in acute hyperammonemia. *Neurochem.Int.*, **41**, 155-160.

Berg, D. J., Zhang, J., Lauricella, D. M. and Moore, S. A. (2001). Il-10 is a central regulator of cyclooxygenase-2 expression and prostaglandin production. *J.Immunol.*, **166**, 2674-2680.

Beuckmann, C. T., Fujimori, K., Urade, Y. and Hayaishi, O. (2000). Identification of mu-class glutathione transferases M2-2 and M3-3 as cytosolic prostaglandin E synthases in the human brain. *Neurochem.Res.*, **25**, 733-738.

Biozzi, G. and Stiffel, C. (1965). The physiopathology of the reticuloendothelial cells of the liver and spleen. *Prog Liver Dis*, **2**, 166-191.

Bishop-Bailey, D., Calatayud, S., Warner, T. D., Hla, T. and Mitchell, J. A. (2002). Prostaglandins and the regulation of tumor growth. *J.Environ.Pathol.Toxicol.Oncol.*, **21**, 93-101.

Bissell, D. M. (1998). Hepatic fibrosis as wound repair: a progress report. *J.Gastroenterol.*, **33**, 295-302.

Blouin, A., Bolender, R. P. and Weibel, E. R. (1977). Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. A stereological study. *J.Cell Biol.*, **72**, 441-455.

Bogdan, C. (2001). Nitric oxide and the immune response. *Nat.Immunol.*, **2**, 907-916.

Bouwens, L., Baekeland, M., De Zanger, R. and Wisse, E. (1986). Quantitation, tissue distribution and proliferation kinetics of Kupffer cells in normal rat liver. *Hepatology*, **6**, 718-722.

Bouwens, L., De Bleser, P., Vanderkerken, K., Geerts, B. and Wisse, E. (1992). Liver cell heterogeneity: functions of non-parenchymal cells. *Enzyme*, **46**, 155-168.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal.Biochem.*, **72**, 248-254.

Braet, F. and Wisse, E. (2002). Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. *Comp Hepatol.*, **1**, 1.

Bredt, D. S., Hwang, P. M. and Snyder, S. H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature*, **347**, 768-770.

Bykov, I., Ylipaasto, P., Eerola, L. and Lindros, K. O. (2004). Functional Differences between Periportal and Perivenous Kupffer Cells Isolated by Digitonin-Collagenase Perfusion. *Comp Hepatol.*, **3 Suppl 1**, S34.

Chandrasekharan, N. V., Dai, H., Roos, K. L., Evanson, N. K., Tomsik, J., Elton, T. S. and Simmons, D. L. (2002). COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc.Natl.Acad.Sci.U.S.A*, **99**, 13926-13931.

Chen, L. Y., Watanabe, K. and Hayaishi, O. (1992). Purification and characterization of prostaglandin F synthase from bovine liver. *Arch.Biochem.Biophys.*, **296**, 17-26.

Christ-Hazelhof, E. and Nugteren, D. H. (1982). Isolation of PGH-PGD isomerase from rat spleen. *Methods Enzymol.*, **86**, 77-84.

Cruz-Gervis, R., Stecenko, A. A., Dworski, R., Lane, K. B., Loyd, J. E., Pierson, R., King, G. and Brigham, K. L. (2002). Altered prostanoid production by fibroblasts cultured from the lungs of human subjects with idiopathic pulmonary fibrosis. *Respir.Res.*, **3**, 17.

Curran, R. D., Billiar, T. R., Stuehr, D. J., Hofmann, K. and Simmons, R. L. (1989). Hepatocytes produce nitrogen oxides from L-arginine in response to inflammatory products of Kupffer cells. *J.Exp.Med.*, **170**, 1769-1774.

Curran, R. D., Billiar, T. R., Stuehr, D. J., Ochoa, J. B., Harbrecht, B. G., Flint, S. G. and Simmons, R. L. (1990). Multiple cytokines are required to induce hepatocyte nitric oxide production and inhibit total protein synthesis. *Ann.Surg.*, **212**, 462-469.

Davies, N. M., Good, R. L., Roupe, K. A. and Yanez, J. A. (2004). Cyclooxygenase-3: axiom, dogma, anomaly, enigma or splice error?--Not as easy as 1, 2, 3. *J.Pharm.Pharm.Sci.*, **7**, 217-226.

Davis, B. H. (1997). Hepatic fibrosis overview and signalling. *Cells of Hepatic Sinusoid*, **6**, 1-4.

Davis, B. H. and Kresina, T. F. (1996). Hepatic fibrogenesis. *Clin.Lab Med.*, **16**, 361-375.

Dayer, J. M. (2002). The saga of the discovery of IL-1 and TNF and their specific inhibitors in the pathogenesis and treatment of rheumatoid arthritis. *Joint Bone Spine*, **69**, 123-132.

Decker, K. (1987). [Function of non-parenchymal cells of the liver]. *Internist (Berl)*, **28**, 420-428.

Decker, K. (1990). Biologically active products of stimulated liver macrophages (Kupffer cells). *Eur.J.Biochem.*, **192**, 245-261.

Decker, K. (1991). Signal paths and regulation of superoxide, eicosanoid and cytokine formation in macrophages of rat liver. *Adv.Exp.Med.Biol.*, **283**, 507-520.

Dennis, E. A. (2000). Phospholipase A2 in eicosanoid generation. *Am.J.Respir.Crit Care Med.*, **161**, S32-S35.

Diaz, B. L. and Arm, J. P. (2003). Phospholipase A(2). *Prostaglandins Leukot.Essent.Fatty Acids*, **69**, 87-97.

Dieckmann-Schuppert, A. and Schnittler, H. J. (1997). A simple assay for quantification of protein in tissue sections, cell cultures, and cell homogenates, and of protein immobilized on solid surfaces. *Cell Tissue Res.*, **288**, 119-126.

Dieter, P. (1996). [Function and Regulation of Eicosanoids in the Liver]. *Die Medizinische Welt*, **47**, 70-73.

Dieter, P., Altin, J. G., Decker, K. and Bygrave, F. L. (1987). Possible involvement of eicosanoids in the zymosan and arachidonic-acid-induced oxygen uptake, glycogenolysis and Ca2+ mobilization in the perfused rat liver. *Eur.J.Biochem.*, **165**, 455-460.

Dieter, P., Ambs, P., Fitzke, E., Hidaka, H., Hoffmann, R. and Schwende, H. (1995). Comparative studies of cytotoxicity and the release of TNF-alpha, nitric oxide, and eicosanoids of liver macrophages treated with lipopolysaccharide and liposome-encapsulated MTP-PE. *J.Immunol.*, **155**, 2595-2604.

Dieter, P. and Fitzke, E. (1993). Formation of diacylglycerol, inositol phosphates, arachidonic acid and its metabolites in macrophages. *Eur.J.Biochem.*, **218**, 753-758.

Dieter, P., Hempel, U., Kamionka, S., Kolada, A., Malessa, B., Fitzke, E. and Tran-Thi, T. A. (1999). Prostaglandin E2 affects differently the release of inflammatory mediators from resident macrophages by LPS and muramyl tripeptides. *Mediators.Inflamm.*, **8**, 295-303.

Dieter, P., Kolada, A., Kamionka, S., Schadow, A. and Kaszkin, M. (2002). Lipopolysaccharide-induced release of arachidonic acid and prostaglandins in liver macrophages: regulation by Group IV cytosolic phospholipase A2, but not by Group V and Group IIA secretory phospholipase A2. *Cell Signal.*, **14**, 199-204.

Dieter, P., Peters, T., Schulze-Specking, A. and Decker, K. (1989). Independent regulation of thromboxane and prostaglandin synthesis in liver macrophages. *Biochem.Pharmacol.*, **38**, 1577-1581.

Dieter, P., Scheibe, R., Bezugla, Y., Matthe, E., Schuch, S., Treffkorn, L., Bernard, B., Kamionka, S. and Kolada, A. (2004). The regulatory role of prostaglandin E2 in liver (patho) physiology is controlled at its site of synthesis and its action on the receptors. *Comp Hepatol.*, **3 Suppl 1**, S35.

Dieter, P., Scheibe, R., Jakobsson, P. J., Watanabe, K., Kolada, A. and Kamionka, S. (2000). Functional coupling of cyclooxygenase 1 and 2 to discrete prostanoid synthases in liver macrophages. *Biochem.Biophys.Res.Commun.*, **276**, 488-492.

Dieter, P., Scheibe, R., Tolzer S., Kamionka, S. and Kolada, A. (2001). Prostaglandin E2, an important regulator of (patho)physiological liver functions: its synthesis and release in Kupffer cells is regulated at multiple levels. *Cells of Hepatic Sinusoid*, **8**, 6-10.

Dieter, P., Schulze-Specking, A. and Decker, K. (1988). Ca2+ requirement of prostanoid but not of superoxide production by rat Kupffer cells. *Eur.J.Biochem.*, **177**, 61-67.

Duyster, J., Hidaka, H., Decker, K. and Dieter, P. (1992). Proteinkinase C beta-isoform triggers the formation of prostanoids and superoxide in liver macrophages. *Biochem.Biophys.Res.Commun.*, **183**, 1247-1253.

Eyhorn, S., Schlayer, H. J., Henninger, H. P., Dieter, P., Hermann, R., Woort-Menker, M., Becker, H., Schaefer, H. E. and Decker, K. (1988). Rat hepatic sinusoidal endothelial cells in monolayer culture. Biochemical and ultrastructural characteristics. *J.Hepatol.*, **6**, 23-35.

Farzaneh-Far, R. and Moore, K. (2001). Nitric oxide and the liver. *Liver*, **21**, 161-174.

Fennekohl, A., Lucas, M. and Puschel, G. P. (2000). Induction by interleukin 6 of G(s)-coupled prostaglandin E(2) receptors in rat hepatocytes mediating a prostaglandin E(2)-dependent inhibition of the hepatocyte's acute phase response. *Hepatology*, **31**, 1128-1134.

Fermor, B., Weinberg, J. B., Pisetsky, D. S., Misukonis, M. A., Fink, C. and Guilak, F. (2002). Induction of cyclooxygenase-2 by mechanical stress through a nitric oxide-regulated pathway. *Osteoarthritis.Cartilage.*, **10**, 792-798.

Fernandez-Morata, J. C., Mullol, J., Fuentes, M., Pujols, L., Roca-Ferrer, J., Perez, M., Xaubet, A. and Picado, C. (2000). Regulation of cyclooxygenase-1 and -2 expression in human nasal mucosa. Effects of cytokines and dexamethasone. *Clin.Exp.Allergy*, **30**, 1275-1284.

Filion, F., Bouchard, N., Goff, A. K., Lussier, J. G. and Sirois, J. (2001). Molecular cloning and induction of bovine prostaglandin E synthase by gonadotropins in ovarian follicles prior to ovulation in vivo. *J.Biol.Chem.*, **276**, 34323-34330.

Fisher, R. A., Robertson, S. M. and Olson, M. S. (1987). Stimulation of glycogenolysis and vasoconstriction in the perfused rat liver by the thromboxane A2 analogue U-46619. *J.Biol.Chem.*, **262**, 4631-4638.

Flower, R. J. (2003). The development of COX2 inhibitors. *Nat.Rev.Drug Discov.*, **2**, 179-191.

Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science*, **294**, 1871-1875.

Gaillard, T., Mulsch, A., Busse, R., Klein, H. and Decker, K. (1991). Regulation of nitric oxide production by stimulated rat Kupffer cells. *Pathobiology*, **59**, 280-283.

Gallo, O., Fabbroni, V., Sardi, I., Magnelli, L., Boddi, V. and Franchi, A. (2002). Correlation between nitric oxide and cyclooxygenase-2 pathways in head and neck squamous cell carcinomas. *Biochem.Biophys.Res.Commun.*, **299**, 517-524.

Gardner, C. R., Heck, D. E., Yang, C. S., Thomas, P. E., Zhang, X. J., DeGeorge, G. L., Laskin, J. D. and Laskin, D. L. (1998). Role of nitric oxide in acetaminophen-induced hepatotoxicity in the rat. *Hepatology*, **27**, 748-754.

Gatmaitan, Z., Varticovski, L., Ling, L., Mikkelsen, R., Steffan, A. M. and Arias, I. M. (1996). Studies on fenestral contraction in rat liver endothelial cells in culture. *Am.J.Pathol.*, **148**, 2027-2041.

Gilchrist, M., Savoie, M., Nohara, O., Wills, F. L., Wallace, J. L. and Befus, A. D. (2002). Nitric oxide synthase and nitric oxide production in in vivo-derived mast cells. *J.Leukoc.Biol.*, **71**, 618-624.

Goda, N., Suzuki, K., Naito, M., Takeoka, S., Tsuchida, E., Ishimura, Y., Tamatani, T. and Suematsu, M. (1998). Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation. *J.Clin.Invest*, **101**, 604-612.

Gomez-Foix, A. M., Rodriguez-Gil, J. E., Guinovart, J. J. and Bosch, F. (1989). Prostaglandins E2 and F2 alpha affect glycogen synthase and phosphorylase in isolated hepatocytes. *Biochem.J.*, **261**, 93-97.

Goss, J. A., Mangino, M. J., Callery, M. P. and Flye, M. W. (1993). Prostaglandin E2 downregulates Kupffer cell production of IL-1 and IL-6 during hepatic regeneration. *Am.J.Physiol*, **264**, G601-G608.

Graupera, M., Garcia-Pagan, J. C., Pares, M., Abraldes, J. G., Rosello, J., Bosch, J. and Rodes, J. (2003). Cyclooxygenase-1 inhibition corrects endothelial dysfunction in cirrhotic rat livers. *J.Hepatol.*, **39**, 515-521.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. and Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal.Biochem.*, **126**, 131-138.

Gross, V., Andus, T., Tran-Thi, T. A., Schwarz, R. T., Decker, K. and Heinrich, P. C. (1983). 1-deoxynojirimycin impairs oligosaccharide processing of alpha 1-proteinase inhibitor and inhibits its secretion in primary cultures of rat hepatocytes. *J.Biol.Chem.*, **258**, 12203-12209.

Hamberg, M., Svensson, J. and Samuelsson, B. (1975). Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc.Natl.Acad.Sci.U.S.A*, **72**, 2994-2998.

Hammarstrom, S. and Falardeau, P. (1977). Resolution of prostaglandin endoperoxide synthase and thromboxane synthase of human platelets. *Proc.Natl.Acad.Sci.U.S.A*, **74**, 3691-3695.

Harbrecht, B. G. and Billiar, T. R. (1995). The role of nitric oxide in Kupffer cell-hepatocyte interactions. *Shock*, **3**, 79-87.

Haurand, M. and Ullrich, V. (1985). Isolation and characterization of thromboxane synthase from human platelets as a cytochrome P-450 enzyme. *J.Biol.Chem.*, **260**, 15059-15067.

Hayashi, H., Fujii, Y., Watanabe, K., Urade, Y. and Hayaishi, O. (1989). Enzymatic conversion of prostaglandin H2 to prostaglandin F2 alpha by aldehyde reductase from human liver: comparison to the prostaglandin F synthetase from bovine lung. *J.Biol.Chem.*, **264**, 1036-1040.

Hecker, M. and Ullrich, V. (1989). On the mechanism of prostacyclin and thromboxane A2 biosynthesis. *J.Biol.Chem.*, **264**, 141-150.

Helliwell, R. J., Adams, L. F. and Mitchell, M. D. (2004). Prostaglandin synthases: recent developments and a novel hypothesis. *Prostaglandins Leukot.Essent.Fatty Acids*, **70**, 101-113.

Hirai, H., Tanaka, K., Yoshie, O., Ogawa, K., Kenmotsu, K., Takamori, Y., Ichimasa, M., Sugamura, K., Nakamura, M., Takano, S. and Nagata, K. (2001). Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J.Exp.Med.*, **193**, 255-261.

Hisama, N., Yamaguchi, Y., Miyanari, N., Ichiguchi, O., Goto, M., Mori, K. and Ogawa, M. (1995). Ischemia-reperfusion injury: the role of Kupffer cells in the production of cytokine-induced neutrophil chemoattractant, a member of the interleukin-8 family. *Transplant.Proc.*, **27**, 1604-1606.

Hoek, J. B. and Pastorino, J. G. (2002). Ethanol, oxidative stress, and cytokine-induced liver cell injury. *Alcohol*, **27**, 63-68.

Hoffmann, A., Conradt, H. S., Gross, G., Nimtz, M., Lottspeich, F. and Wurster, U. (1993). Purification and chemical characterization of beta-trace protein from human cerebrospinal fluid: its identification as prostaglandin D synthase. *J.Neurochem.*, **61**, 451-456.

Horton, R. A., Ceppi, E. D., Knowles, R. G. and Titheradge, M. A. (1994). Inhibition of hepatic gluconeogenesis by nitric oxide: a comparison with endotoxic shock. *Biochem.J.*, **299 (Pt 3)**, 735-739.

Hsu, P. Y., Tsai, A. L., Kulmacz, R. J. and Wang, L. H. (1999). Expression, purification, and spectroscopic characterization of human thromboxane synthase. *J.Biol.Chem.*, **274**, 762-769.

Hu, J., Meng, Q., Roy, S. K., Raha, A., Hu, J., Zhang, J., Hashimoto, K. and Kalvakolanu, D. V. (2002). A novel transactivating factor that regulates interferon-gamma-dependent gene expression. *J.Biol.Chem.*, **277**, 30253-30263.

Ignarro, L. J. (1990). Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu.Rev.Pharmacol.Toxicol.*, **30**, 535-560.

Jakobsson, P. J., Morgenstern, R., Mancini, J., Ford-Hutchinson, A. and Persson, B. (2000). Membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). A widespread protein superfamily. *Am.J.Respir.Crit Care Med.*, **161**, S20-S24.

Jakobsson, P. J., Morgenstern, R., Mancini, J., Ford-Hutchinson, A. and Persson, B. (1999a). Common structural features of MAPEG -- a widespread superfamily of membrane associated proteins with highly divergent functions in eicosanoid and glutathione metabolism. *Protein Sci.*, **8**, 689-692.

Jakobsson, P. J., Thoren, S., Morgenstern, R. and Samuelsson, B. (1999b). Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc.Natl.Acad.Sci.U.S.A*, **96**, 7220-7225.

Johnson, S. J. (1996). Extracellular matrix proteins and hepatic fibrosis. *Hepatogastroenterology*, **43**, 44-55.

Jones, B. E. and Czaja, M. J. (1998). III. Intracellular signaling in response to toxic liver injury. *Am.J.Physiol*, **275**, G874-G878.

Jones, D. A. and Fitzpatrick, F. A. (1990). "Suicide" inactivation of thromboxane A2 synthase. Characteristics of mechanism-based inactivation with isolated enzyme and intact platelets. *J.Biol.Chem.*, **265**, 20166-20171.

Ju, C., Reilly, T. P., Bourdi, M., Radonovich, M. F., Brady, J. N., George, J. W. and Pohl, L. R. (2002). Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chem.Res.Toxicol.*, **15**, 1504-1513.

Kahlke, V., Dohm, C., Mees, T., Brotzmann, K., Schreiber, S. and Schroder, J. (2002). Early interleukin-10 treatment improves survival and enhances immune function only in males after hemorrhage and subsequent sepsis. *Shock*, **18**, 24-28.

Kanaoka, Y. and Urade, Y. (2003). Hematopoietic prostaglandin D synthase. *Prostaglandins Leukot.Essent.Fatty Acids*, **69**, 163-167.

Karck, U., Peters, T. and Decker, K. (1988). The release of tumor necrosis factor from endotoxin-stimulated rat Kupffer cells is regulated by prostaglandin E2 and dexamethasone. *J.Hepatol.*, **7**, 352-361.

Katagiri, H., Ito, Y., Ishii, K., Hayashi, I., Suematsu, M., Yamashina, S., Murata, T., Narumiya, S., Kakita, A. and Majima, M. (2004). Role of thromboxane derived from COX-1 and -2 in hepatic microcirculatory dysfunction during endotoxemia in mice. *Hepatology*, **39**, 139-150.

Kawada N. (1999). Regulation of hepatic gene expression by Kupffer cells-derived mediators. *Cells of Hepatic Sinusoid*, **7**, 251-256.

Kawada, N., Klein, H. and Decker, K. (1992). Eicosanoid-mediated contractility of hepatic stellate cells. *Biochem.J.*, **285 (Pt 2)**, 367-371.

Kershenobich, S. D. and Weissbrod, A. B. (2003). Liver fibrosis and inflammation. A review. *Ann.Hepatol.*, **2**, 159-163.

Kis, B., Snipes, A., Bari, F. and Busija, D. W. (2004). Regional distribution of cyclooxygenase-3 mRNA in the rat central nervous system. *Brain Res.Mol.Brain Res.*, **126**, 78-80.

Klebe, R. J., Rodriguez, S. A., VerBeek, M. L. and Giambernardi, T. A. (1999). Simple method for "hot-starting" RT-PCR. *Biotechniques*, **27**, 1108-1110.

Knook, D. L., Blansjaar, N. and Sleyster, E. C. (1977). Isolation and characterization of Kupffer and endothelial cells from the rat liver. *Exp.Cell Res.*, **109**, 317-329.

Kobayashi, T., Nakatani, Y., Tanioka, T., Tsujimoto, M., Nakajo, S., Nakaya, K., Murakami, M. and Kudo, I. (2004). Regulation of cytosolic prostaglandin E synthase by phosphorylation. *Biochem.J.*, **381**, 59-69.

Kojima, Y., Suzuki, S., Tsuchiya, Y., Konno, H., Baba, S. and Nakamura, S. (2003). Regulation of pro-inflammatory and anti-inflammatory cytokine responses by Kupffer cells in endotoxin-enhanced reperfusion injury after total hepatic ischemia. *Transpl.Int.*, **16**, 231-240.

Kroncke, K. D., Fehsel, K. and Kolb-Bachofen, V. (1997). Nitric oxide: cytotoxicity versus cytoprotection--how, why, when, and where? *Nitric.Oxide.*, **1**, 107-120.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Lazarus, M., Kubata, B. K., Eguchi, N., Fujitani, Y., Urade, Y. and Hayaishi, O. (2002a). Biochemical characterization of mouse microsomal prostaglandin E synthase-1 and its colocalization with cyclooxygenase-2 in peritoneal macrophages. *Arch.Biochem.Biophys.*, **397**, 336-341.

Lazarus, M., Munday, C. J., Eguchi, N., Matsumoto, S., Killian, G. J., Kubata, B. K. and Urade, Y. (2002b). Immunohistochemical localization of microsomal PGE synthase-1 and cyclooxygenases in male mouse reproductive organs. *Endocrinology*, **143**, 2410-2419.

Leifeld, L., Fielenbach, M., Dumoulin, F. L., Speidel, N., Sauerbruch, T. and Spengler, U. (2002). Inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) expression in fulminant hepatic failure. *J.Hepatol.*, **37**, 613-619.

Lemieux, L. I., Rahal, S. S. and Kennedy, C. R. (2002). Prostaglandin E2 Reduces Arachidonic Acid Release in Murine Podocytes; Evidence for an Autocrine Feedback Loop. *Am.J.Physiol Cell Physiol*,

Leslie, C. C. (2004). Regulation of the specific release of arachidonic acid by cytosolic phospholipase A2. *Prostaglandins Leukot.Essent.Fatty Acids*, **70**, 373-376.

Li, J. and Billiar, T. R. (1999). The anti-apoptotic actions of nitric oxide in hepatocytes. *Cell Death.Differ.*, **6**, 952-955.

Lirk, P., Hoffmann, G. and Rieder, J. (2002). Inducible nitric oxide synthase--time for reappraisal. *Curr.Drug Targets.Inflamm.Allergy*, **1**, 89-108.

Liston, T. E. and Roberts, L. J. (1985). Transformation of prostaglandin D2 to 9 alpha, 11 beta-(15S)-trihydroxyprosta-(5Z,13E)-dien-1-oic acid (9 alpha, 11 beta-prostaglandin F2): a unique biologically active prostaglandin produced enzymatically in vivo in humans. *Proc.Natl.Acad.Sci.U.S.A*, **82**, 6030-6034.

Liu, T. H., Robinson, E. K., Helmer, K. S., West, S. D., Castaneda, A. A., Chang, L. and Mercer, D. W. (2002). Does upregulation of inducible nitric oxide synthase play a role in hepatic injury? *Shock*, **18**, 549-554.

Madden, T. L., Tatusov, R. L. and Zhang, J. (1996). Applications of network BLAST server. *Methods Enzymol.*, **266**, 131-141.

Malik, R., Selden, C. and Hodgson, H. (2002). The role of non-parenchymal cells in liver growth. *Semin.Cell Dev.Biol.*, **13**, 425-431.

Mancini, J. A., Blood, K., Guay, J., Gordon, R., Claveau, D., Chan, C. C. and Riendeau, D. (2001). Cloning, expression, and up-regulation of inducible rat prostaglandin e synthase during lipopolysaccharide-induced pyresis and adjuvant-induced arthritis. *J.Biol.Chem.*, **276**, 4469-4475.

F. Marks and Fьrstenberg G.(ed.) (1999). *Prostaglandins, Leukotrienes and Other Eicosanoids*. Wiley-VCH, Weinheim, 47-108.

Masferrer, J. L. and Seibert, K. (1994). Regulation of prostaglandin synthesis by glucocorticoids. *Receptor*, **4**, 25-30.

Matsumura, H., Takahata, R. and Hayaishi, O. (1991). Inhibition of sleep in rats by inorganic selenium compounds, inhibitors of prostaglandin D synthase. *Proc.Natl.Acad.Sci.U.S.A*, **88**, 9046-9050.

McCall, T. B., Boughton-Smith, N. K., Palmer, R. M., Whittle, B. J. and Moncada, S. (1989). Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochem.J.*, **261**, 293-296.

Mellion, B. T., Ignarro, L. J., Ohlstein, E. H., Pontecorvo, E. G., Hyman, A. L. and Kadowitz, P. J. (1981). Evidence for the inhibitory role of guanosine 3', 5'-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. *Blood*, **57**, 946-955.

Mene, P. and Dunn, M. J. (1986). Contractile effects of TxA2 and endoperoxide analogues on cultured rat glomerular mesangial cells. *Am.J.Physiol*, **251**, F1029-F1035.

Meyer, D. J., Muimo, R., Thomas, M., Coates, D. and Isaac, R. E. (1996). Purification and characterization of prostaglandin-H E-isomerase, a sigma-class glutathione S-transferase, from Ascaridia galli. *Biochem.J.*, **313 (Pt 1)**, 223-227.

Miethke, A., Feussner, M., Planitzer, G., Richter, H., Gutsmann, M. and Gossrau, R. (2003). Localization of NOS-1 in the sarcolemma region of a subpopulation of atrial cardiomyocytes including myoendocrine cells and NOS-3 in vascular and endocardial endothelial cells of the rat heart. *Acta Histochem.*, **105**, 43-55.

Mitkov, D. (1990). Effect of prostaglandin E2 on urea synthesis and hepatic hemodynamics in rat livers. An in vivo/in vitro study. *Prostaglandins Leukot.Essent.Fatty Acids*, **41**, 279-283.

Moncada, S., Needleman, P., Bunting, S. and Vane, J. R. (1976). Prostaglandin endoperoxide and thromboxane generating systems and their selective inhibition. *Prostaglandins*, **12**, 323-335.

Moncada, S., Palmer, R. M. and Higgs, E. A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol.Rev.*, **43**, 109-142.

Moonen, P., Buytenhek, M. and Nugteren, D. H. (1982). Purification of PGH-PGE isomerase from sheep vesicular glands. *Methods Enzymol.*, **86**, 84-91.

Murakami, M. and Kudo, I. (2004). Recent advances in molecular biology and physiology of the prostaglandin E2-biosynthetic pathway. *Prog.Lipid Res.*, **43**, 3-35.

Murakami, M., Naraba, H., Tanioka, T., Semmyo, N., Nakatani, Y., Kojima, F., Ikeda, T., Fueki, M., Ueno, A., Oh, S. and Kudo, I. (2000). Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. *J.Biol.Chem.*, **275**, 32783-32792.

Muriel, P. (2000). Regulation of nitric oxide synthesis in the liver. *J.Appl.Toxicol.*, **20**, 189-195.

Nagano, S., Otsuka, T., Niiro, H., Yamaoka, K., Arinobu, Y., Ogami, E., Akahoshi, M., Inoue, Y., Miyake, K., Nakashima, H., Niho, Y. and Harada, M. (2002). Molecular mechanisms of lipopolysaccharide-induced cyclooxygenase-2 expression in human neutrophils: involvement of the mitogen-activated protein kinase pathway and regulation by anti-inflammatory cytokines. *Int.Immunol.*, **14**, 733-740.

Naraba, H., Murakami, M., Matsumoto, H., Shimbara, S., Ueno, A., Kudo, I. and Oh-Ishi, S. (1998). Segregated coupling of phospholipases A2, cyclooxygenases, and terminal prostanoid synthases in different phases of prostanoid biosynthesis in rat peritoneal macrophages. *J.Immunol.*, **160**, 2974-2982.

Naraba, H., Yokoyama, C., Tago, N., Murakami, M., Kudo, I., Fueki, M., Oh-Ishi, S. and Tanabe, T. (2002). Transcriptional regulation of the membrane-associated prostaglandin E2 synthase gene. Esential role of the transcription factor EGR-1. *J.Biol.Chem.*,

Needleman, P., Moncada, S., Bunting, S., Vane, J. R., Hamberg, M. and Samuelsson, B. (1976). Identification of an enzyme in platelet microsomes which generates thromboxane A2 from prostaglandin endoperoxides. *Nature*, **261**, 558-560.

Nelson, D. R., Tu, Z., Soldevila-Pico, C., Abdelmalek, M., Zhu, H., Xu, Y. L., Cabrera, R., Liu, C. and Davis, G. L. (2003). Long-term interleukin 10 therapy in chronic hepatitis C patients has a proviral and anti-inflammatory effect. *Hepatology*, **38**, 859-868.

Niiro, H., Otsuka, T., Tanabe, T., Hara, S., Kuga, S., Nemoto, Y., Tanaka, Y., Nakashima, H., Kitajima, S., Abe, M. and . (1995). Inhibition by interleukin-10 of inducible cyclooxygenase expression in lipopolysaccharide-stimulated monocytes: its underlying mechanism in comparison with interleukin-4. *Blood*, **85**, 3736-3745.

Nishida, J., McCuskey, R. S., McDonnell, D. and Fox, E. S. (1994). Protective role of NO in hepatic microcirculatory dysfunction during endotoxemia. *Am.J.Physiol*, **267**, G1135-G1141.

Nunez, A., Gomez-Villamandos, J. C., Sanchez-Cordon, P. J., Fernandez, d. M., Pedrera, M., Salguero, F. J. and Carrasco, L. (2005). Expression of proinflammatory cytokines by hepatic macrophages in acute classical swine fever. *J.Comp Pathol.*, **133**, 23-32.

Nusing, R., Schneider-Voss, S. and Ullrich, V. (1990). Immunoaffinity purification of human thromboxane synthase. *Arch.Biochem.Biophys.*, **280**, 325-330.

Nussler, A. K. and Billiar, T. R. (1993). Inflammation, immunoregulation, and inducible nitric oxide synthase. *J.Leukoc.Biol.*, **54**, 171-178.

Ogletree, M. L. (1987). Overview of physiological and pathophysiological effects of thromboxane A2. *Fed.Proc.*, **46**, 133-138.

Ogorochi, T., Ujihara, M. and Narumiya, S. (1987). Purification and properties of prostaglandin H-E isomerase from the cytosol of human brain: identification as anionic forms of glutathione S-transferase. *J.Neurochem.*, **48**, 900-909.

Oguro, T., Takahashi, Y., Ashino, T., Takaki, A., Shioda, S., Horai, R., Asano, M., Sekikawa, K., Iwakura, Y. and Yoshida, T. (2002). Involvement of tumor necrosis factor alpha, rather than interleukin-1alpha/beta or nitric oxides in the heme oxygenase-1 gene expression by lipopolysaccharide in the mouse liver. *FEBS Lett.*, **516**, 63-66.

Ohashi, K., Ruan, K. H., Kulmacz, R. J., Wu, K. K. and Wang, L. H. (1992). Primary structure of human thromboxane synthase determined from the cDNA sequence. *J.Biol.Chem.*, **267**, 789-793.

Olinga, P., Merema, M. T., De Jager, M. H., Derks, F., Melgert, B. N., Moshage, H., Slooff, M. J., Meijer, D. K., Poelstra, K. and Groothuis, G. M. (2001). Rat liver slices as a tool to study LPS-induced inflammatory response in the liver. *J.Hepatol.*, **35**, 187-194.

Ou, J., Carlos, T. M., Watkins, S. C., Saavedra, J. E., Keefer, L. K., Kim, Y. M., Harbrecht, B. G. and Billiar, T. R. (1997). Differential effects of nonselective nitric oxide synthase (NOS) and selective inducible NOS inhibition on hepatic necrosis, apoptosis, ICAM-1 expression, and neutrophil accumulation during endotoxemia. *Nitric.Oxide.*, **1**, 404-416.

Ouellet, M., Falgueyret, J. P., Ear, P. H., Pen, A., Mancini, J. A., Riendeau, D. and Percival, M. D. (2002). Purification and characterization of recombinant microsomal prostaglandin E synthase-1. *Protein Expr.Purif.*, **26**, 489-495.

Perkins, D. J. and Kniss, D. A. (1999). Blockade of nitric oxide formation down-regulates cyclooxygenase-2 and decreases PGE2 biosynthesis in macrophages. *J.Leukoc.Biol.*, **65**, 792-799.

Pinzani, M. and Marra, F. (2001). Cytokine receptors and signaling in hepatic stellate cells. *Semin.Liver Dis.*, **21**, 397-416.

Pollock, J. S., Forstermann, U., Tracey, W. R. and Nakane, M. (1995). Nitric oxide synthase isozymes antibodies. *Histochem.J.*, **27**, 738-744.

Refsnes, M., Thoresen, G. H., Dajani, O. F. and Christoffersen, T. (1994). Stimulation of hepatocyte DNA synthesis by prostaglandin E2 and prostaglandin F2 alpha: additivity with the effect of norepinephrine, and synergism with epidermal growth factor. *J.Cell Physiol*, **159**, 35-40.

Rieder, H., Armbrust, T., Meyer zum Buschenfelde, K. H. and Ramadori, G. (1993). Contribution of sinusoidal endothelial liver cells to liver fibrosis: expression of transforming growth factor-beta 1 receptors and modulation of plasmin-generating enzymes by transforming growth factor-beta 1. *Hepatology*, **18**, 937-944.

Romero, L. I., Tatro, J. B., Field, J. A. and Reichlin, S. (1996). Roles of IL-1 and TNF-alpha in endotoxin-induced activation of nitric oxide synthase in cultured rat brain cells. *Am.J.Physiol*, **270**, R326-R332.

Rozen S. and Skaletsky H. (2000). *Primer3 on the WWW for general users and for biologist programmers*. Humana Press, Totowa, NJ. 365-386.

Sato, T. A., Keelan, J. A. and Mitchell, M. D. (2003). Critical paracrine interactions between TNF-alpha and IL-10 regulate lipopolysaccharide-stimulated human choriodecidual cytokine and prostaglandin E2 production. *J.Immunol.*, **170**, 158-166.

Seibert, K., Sheller, J. R. and Roberts, L. J. (1987). (5Z,13E)-(15S)-9 alpha,11 beta,15-trihydroxyprosta-5,13-dien-1-oic acid (9 alpha,11 beta-prostaglandin F2): formation and metabolism by human lung and contractile effects on human bronchial smooth muscle. *Proc.Natl.Acad.Sci.U.S.A*, **84**, 256-260.

Shen, R. F. and Tai, H. H. (1986). Immunoaffinity purification and characterization of thromboxane synthase from porcine lung. *J.Biol.Chem.*, **261**, 11592-11599.

Shen, R. F. and Tai, H. H. (1998). Thromboxanes: synthase and receptors. *J.Biomed.Sci.*, **5**, 153-172.

Shen, R. F., Zhang, L., Baek, S. J., Tai, H. H. and Lee, K. D. (1994). The porcine thromboxane synthase-encoding cDNA: sequence, mRNA expression and enzyme production in Sf9 insect cells. *Gene*, **140**, 261-265.

Shimizu, T., Yamamoto, S. and Hayaishi, O. (1979). Purification and properties of prostaglandin D synthetase from rat brain. *J.Biol.Chem.*, **254**, 5222-5228.

Shinomiya, S., Naraba, H., Ueno, A., Utsunomiya, I., Maruyama, T., Ohuchida, S., Ushikubi, F., Yuki, K., Narumiya, S., Sugimoto, Y., Ichikawa, A. and Oh-Ishi, S. (2001). Regulation of TNFalpha and interleukin-10 production by prostaglandins I(2) and E(2): studies with prostaglandin receptor-deficient mice and prostaglandin E-receptor subtype-selective synthetic agonists. *Biochem.Pharmacol.*, **61**, 1153-1160.

Sleyster, E. C. and Knook, D. L. (1982). Relation between localization and function of rat liver Kupffer cells. *Lab Invest*, **47**, 484-490.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal.Biochem.*, **150**, 76-85.

Smith, W. L., Dewitt, D. L. and Garavito, R. M. (2000). Cyclooxygenases: structural, cellular, and molecular biology. *Annu.Rev.Biochem.*, **69**, 145-182.

Sokol, R. J. (2002). Liver cell injury and fibrosis. *J.Pediatr.Gastroenterol.Nutr.*, **35 Suppl 1**, S7-10.

Stadler, J., Harbrecht, B. G., Di Silvio, M., Curran, R. D., Jordan, M. L., Simmons, R. L. and Billiar, T. R. (1993). Endogenous nitric oxide inhibits the synthesis of cyclooxygenase products and interleukin-6 by rat Kupffer cells. *J.Leukoc.Biol.*, **53**, 165-172.

Stichtenoth, D. O., Thoren, S., Bian, H., Peters-Golden, M., Jakobsson, P. J. and Crofford, L. J. (2001). Microsomal prostaglandin E synthase is regulated by proinflammatory cytokines and glucocorticoids in primary rheumatoid synovial cells. *J.Immunol.*, **167**, 469-474.

Sun, F. F. (1977). Biosynthesis of thromboxanes in human platelets. I. Characterization and assay of thromboxane synthetase. *Biochem.Biophys.Res.Commun.*, **74**, 1432-1440.

Suzuki, T., Fujii, Y., Miyano, M., Chen, L. Y., Takahashi, T. and Watanabe, K. (1999). cDNA cloning, expression, and mutagenesis study of liver-type prostaglandin F synthase. *J.Biol.Chem.*, **274**, 241-248.

Suzuki-Yamamoto, T., Nishizawa, M., Fukui, M., Okuda-Ashitaka, E., Nakajima, T., Ito, S. and Watanabe, K. (1999a). cDNA cloning, expression and characterization of human prostaglandin F synthase. *FEBS Lett.*, **462**, 335-340.

Suzuki-Yamamoto, T., Yokoi, H., Tsuruo, Y., Watanabe, K. and Ishimura, K. (1999b). Identification of prostaglandin F-producing cells in the liver. *Histochem.Cell Biol.*, **112**, 451-456.

Takeishi, T., Hirano, K., Kobayashi, T., Hasegawa, G., Hatakeyama, K. and Naito, M. (1999). The role of Kupffer cells in liver regeneration. *Arch.Histol.Cytol.*, **62**, 413-422.

Tanaka, T., Urade, Y., Kimura, H., Eguchi, N., Nishikawa, A. and Hayaishi, O. (1997). Lipocalin-type prostaglandin D synthase (beta-trace) is a newly recognized type of retinoid transporter. *J.Biol.Chem.*, **272**, 15789-15795.

Tanaka, Y., Ward, S. L. and Smith, W. L. (1987). Immunochemical and kinetic evidence for two different prostaglandin H-prostaglandin E isomerases in sheep vesicular gland microsomes. *J.Biol.Chem.*, **262**, 1374-1381.

Tanikawa, N., Ohmiya, Y., Ohkubo, H., Hashimoto, K., Kangawa, K., Kojima, M., Ito, S. and Watanabe, K. (2002). Identification and characterization of a novel type of membrane-associated prostaglandin E synthase. *Biochem.Biophys.Res.Commun.*, **291**, 884-889.

Tanioka, T., Nakatani, Y., Kobayashi, T., Tsujimoto, M., Oh-Ishi, S., Murakami, M. and Kudo, I. (2003). Regulation of cytosolic prostaglandin E2 synthase by 90-kDa heat shock protein. *Biochem.Biophys.Res.Commun.*, **303**, 1018-1023.

Tanioka, T., Nakatani, Y., Semmyo, N., Murakami, M. and Kudo, I. (2000). Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2 biosynthesis. *J.Biol.Chem.*, **275**, 32775-32782.

Thompson, K., Maltby, J., Fallowfield, J., McAulay, M., Millward-Sadler, H. and Sheron, N. (1998). Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis. *Hepatology*, **28**, 1597-1606.

Thoren, S. and Jakobsson, P. J. (2000). Coordinate up- and down-regulation of glutathione-dependent prostaglandin E synthase and cyclooxygenase-2 in A549 cells. Inhibition by NS-398 and leukotriene C4. *Eur.J.Biochem.*, **267**, 6428-6434.

Thoren, S., Weinander, R., Saha, S., Jegerschold, C., Pettersson, P. L., Samuelsson, B., Hebert, H., Hamberg, M., Morgenstern, R. and Jakobsson, P. J. (2003). Human microsomal prostaglandin E synthase-1: purification, functional characterization, and projection structure determination. *J.Biol.Chem.*, **278**, 22199-22209.

Tilley, S. L., Coffman, T. M. and Koller, B. H. (2001). Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. *J.Clin.Invest*, **108**, 15-23.

Tone, Y., Miyata, A., Hara, S., Yukawa, S. and Tanabe, T. (1994). Abundant expression of thromboxane synthase in rat macrophages. *FEBS Lett.*, **340**, 241-244.

Tran-Thi, T. A., Decker, K. and Baeuerle, P. A. (1995). Differential activation of transcription factors NF-kappa B and AP-1 in rat liver macrophages. *Hepatology*, **22**, 613-619.

Tran-Thi, T. A., Gyufko, K., Henninger, H., Busse, R. and Decker, K. (1987). Studies on synthesis and degradation of eicosanoids by rat hepatocytes in primary culture. *J.Hepatol.*, **5**, 322-331.

Treffkorn, L., Scheibe, R., Maruyama, T. and Dieter, P. (2004). PGE2 exerts its effect on the LPS-induced release of TNF-alpha, ET-1, IL-1alpha, IL-6 and IL-10 via the EP2 and EP4 receptor in rat liver macrophages. *Prostaglandins Other Lipid Mediat.*, **74**, 113-123.

Tsukamoto, H. (1999). Cytokine regulation of hepatic stellate cells in liver fibrosis. *Alcohol Clin.Exp.Res.*, **23**, 911-916.

Tsutsui, H., Matsui, K., Kawada, N., Hyodo, Y., Hayashi, N., Okamura, H., Higashino, K. and Nakanishi, K. (1997). IL-18 accounts for both TNF-alpha- and Fas ligand-mediated hepatotoxic pathways in endotoxin-induced liver injury in mice. *J.Immunol.*, **159**, 3961-3967.

Tsutsumi, E., Takeuchi, K., Abe, T., Takahashi, N., Kato, T., Taniyama, Y., Ikeda, Y., Ito, S. and Abe, K. (1997). Rat kidney thromboxane synthase: cDNA cloning and gene expression regulation in hydronephrotic kidney. *Prostaglandins*, **53**, 423-431.

Uematsu, S., Matsumoto, M., Takeda, K. and Akira, S. (2002). Lipopolysaccharide-dependent prostaglandin E(2) production is regulated by the glutathione-dependent prostaglandin E(2) synthase gene induced by the Toll-like receptor 4/MyD88/NF-IL6 pathway. *J.Immunol.*, **168**, 5811-5816.

Ueno, R., Ishikawa, Y., Nakayama, T. and Hayaishi, O. (1982). Prostaglandin D2 induces sleep when microinjected into the preoptic area of conscious rats. *Biochem.Biophys.Res.Commun.*, **109**, 576-582.

Urade, Y. and Eguchi, N. (2002). Lipocalin-type and hematopoietic prostaglandin D synthases as a novel example of functional convergence. *Prostaglandins Other Lipid Mediat.*, **68-69**, 375-382.

Urade, Y., Fujimoto, N., Ujihara, M. and Hayaishi, O. (1987). Biochemical and immunological characterization of rat spleen prostaglandin D synthetase. *J.Biol.Chem.*, **262**, 3820-3825.

Urade, Y., Nagata, A., Suzuki, Y., Fujii, Y. and Hayaishi, O. (1989). Primary structure of rat brain prostaglandin D synthetase deduced from cDNA sequence. *J.Biol.Chem.*, **264**, 1041-1045.

Vane, J. R., Bakhle, Y. S. and Botting, R. M. (1998). Cyclooxygenases 1 and 2. *Annu.Rev.Pharmacol.Toxicol.*, **38**, 97-120.

Vos, T. A., Gouw, A. S., Klok, P. A., Havinga, R., van Goor, H., Huitema, S., Roelofsen, H., Kuipers, F., Jansen, P. L. and Moshage, H. (1997). Differential effects of nitric oxide synthase inhibitors on endotoxin-induced liver damage in rats. *Gastroenterology*, **113**, 1323-1333.

Wang, G. S. and Liu, G. T. (1995). Role of nitric oxide in immunological liver damage in mice. *Biochem.Pharmacol.*, **49**, 1277-1281.

Wang, L. H. and Kulmacz, R. J. (2002). Thromboxane synthase: structure and function of protein and gene. *Prostaglandins Other Lipid Mediat.*, **68-69**, 409-422.

Watanabe, K. (2002). Prostaglandin F synthase. *Prostaglandins Other Lipid Mediat.*, **68-69**, 401-407.

Watanabe, K., Fujii, Y., Ohkubo, H., Kuramitsu, S., Kagamiyama, H., Nakanishi, S. and Hayaishi, O. (1991). Expression of bovine lung prostaglandin F synthase in Escherichia coli. *Biochem.Biophys.Res.Commun.*, **181**, 272-278.

Watanabe, K., Kurihara, K. and Suzuki, T. (1999). Purification and characterization of membrane-bound prostaglandin E synthase from bovine heart. *Biochim.Biophys.Acta*, **1439**, 406-414.

Watanabe, K., Kurihara, K., Tokunaga, Y. and Hayaishi, O. (1997). Two types of microsomal prostaglandin E synthase: glutathione-dependent and -independent prostaglandin E synthases. *Biochem.Biophys.Res.Commun.*, **235**, 148-152.

Watanabe, K., Yoshida, R., Shimizu, T. and Hayaishi, O. (1985). Enzymatic formation of prostaglandin F2 alpha from prostaglandin H2 and D2. Purification and properties of prostaglandin F synthetase from bovine lung. *J.Biol.Chem.*, **260**, 7035-7041.

Watkins, D. N., Garlepp, M. J. and Thompson, P. J. (1997). Regulation of the inducible cyclo-oxygenase pathway in human cultured airway epithelial (A549) cells by nitric oxide. *Br.J.Pharmacol.*, **121**, 1482-1488.

Ways, D. K., Cook, P. P., Webster, C. and Parker, P. J. (1992). Effect of phorbol esters on protein kinase C-zeta. *J.Biol.Chem.*, **267**, 4799-4805.

White, D. M., Mikol, D. D., Espinosa, R., Weimer, B., Le Beau, M. M. and Stefansson, K. (1992). Structure and chromosomal localization of the human gene for a brain form of prostaglandin D2 synthase. *J.Biol.Chem.*, **267**, 23202-23208.

E. Wisse, Knook, D. L. and Fraser, R. (1999). *Cells of the Hepatic Sinusoid*. The Netherland,

Yamagata, K., Matsumura, K., Inoue, W., Shiraki, T., Suzuki, K., Yasuda, S., Sugiura, H., Cao, C., Watanabe, Y. and Kobayashi, S. (2001). Coexpression of microsomal-type prostaglandin E synthase with cyclooxygenase-2 in brain endothelial cells of rats during endotoxin-induced fever. *J.Neurosci.*, **21**, 2669-2677.

Yokoyama, C., Miyata, A., Ihara, H., Ullrich, V. and Tanabe, T. (1991). Molecular cloning of human platelet thromboxane A synthase. *Biochem.Biophys.Res.Commun.*, **178**, 1479-1484.

Yokoyama, C., Miyata, A., Suzuki, K., Nishikawa, Y., Yoshimoto, T., Yamamoto, S., Nusing, R., Ullrich, V. and Tanabe, T. (1993). Expression of human thromboxane synthase using a baculovirus system. *FEBS Lett.*, **318**, 91-94.

Yokoyama, Y., Xu, H., Kresge, N., Keller, S., Sarmadi, A. H., Baveja, R., Clemens, M. G. and Zhang, J. X. (2003). Role of thromboxane A2 in early BDL-induced portal hypertension. *Am.J.Physiol Gastrointest.Liver Physiol*, **284**, G453-G460.

Zhang, L., Chase, M. B. and Shen, R. F. (1993). Molecular cloning and expression of murine thromboxane synthase. *Biochem.Biophys.Res.Commun.*, **194**, 741-748.

Zhang, Y., Schneider, A., Rao, R., Lu, W. J., Fan, X., Davis, L., Breyer, R. M., Breyer, M. D. and Guan, Y. (2003). Genomic structure and genitourinary expression of mouse cytosolic prostaglandin E(2) synthase gene. *Biochim.Biophys.Acta*, **1634**, 15-23.

Zhang, Z., Schwartz, S., Wagner, L. and Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *J.Comput.Biol.*, **7**, 203-214.

Acknowledgments

I would like to express my gratitude to those people who made this thesis possible and enriched my life in the past few years.

I'd like to thank my supervisor Prof. Dr. Karl-Heinz van Pée for giving me an opportunity to perform this work.

My special thanks are addressed to Prof. Dr. Peter Dieter for offering me the position in his group and for supporting me during the work on this dissertation.

I also want to thank Dr. Roland Scheibe and Dr. Ute Hempel for their theoretical and practical support and their friendly cooperation. Many thanks belong also to Prof. Dr. med. Ursula Ravens for her encouragement.

I will take this opportunity to thank all members of the Institute of Physiological Chemistry who have contributed to make my stay effective. I cannot list here all the names, I just would like to mention Angelika Kolada, Sabine Kamionka and Brigitte Bernard and give my thanks for their excellent technical assistance. Many thanks belong also to Dr. Dagmar Seidel, Anett Hientzsch and Andrea Stephan for their friendly encouragement.

I have to thank my husband Dr. Viktor Bezugly for his warm support and endurance. He spent a lot of patience and time during my work and gave me a motivation I needed to write my thesis.

I also have to thank my parents and my parent-in law who always supported and encouraged me and especially my mother, my mother-in law and my aunt Galyna for giving me the childfree time to write my thesis.

CURRICULUM VITAE

Name	Yevgeniya Bezugla, geb. Pismenna
Geburtstag, -ort:	05.05.1976 in Pawlograd, Ukraine
Familienstand:	Verheiratet, drei Söhne
1983-1993	Charkow Lyzeum für Physik und Mathematik
1993-1998	Ukrainische pharmazeutische Akademie (Charkiw, Ukraine) Fach: Industriepharmazie
1998-2000	Ukrainische pharmazeutische Akademie (Charkiw, Ukraine) Wissenschaftliche Tätigkeit
2000-2005	Ausführung der Dissertation am Institut für Physiologische Chemie der Technischen Universität Dresden unter wissenschaftlicher Betreuung von Herrn Prof. Dr. K.-H. van Pée und von Herrn Prof. Dr. P. Dieter

Versicherung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.
Die vorliegende Arbeit wurde am Institut für Physiologische Chemie der TU Dresden unter der wissenschaftlichen Betreuung von Herrn Prof. Dr. K.-H. van Pée und Herrn Prof. Dr. Peter Dieter angefertigt.

Dresden, den 16. Juli 2007

Yevgeniya Bezugla